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A Molecular Approach to Understanding the Effects of Original-XPC on the Modulation of the Cecal Microbiota and the Survival of Salmonella in the Poultry Host

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Molecular Approach to Understanding the Effects of Original-XPC^{TM} on the Modulation of the Cecal Microbiota and the Survival of *Salmonella* in the Poultry Host

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by

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Abstract

Salmonella is a foodborne pathogen commonly associated with poultry products; the economic burden to the U.S. is estimated to be approximately $693 million (without factoring in contaminated eggs). Recently, there has been pressure to withdraw sub-therapeutic levels of antibiotics (also known as antibiotic growth promoters; AGP) from poultry due to concern over antibiotic-resistant bacteria spreading to the human population. Therefore, various feed additives have been researched for their ability in providing protection against harmful pathogens and their potential growth promoting effects, both of which were attributes of poultry treated with AGP. This thesis consists of a comprehensive literature review that covers prebiotics and similar compounds, along with their effects on the microbiome. Additionally, the research described in this thesis utilized an *in vitro* anaerobic mixed culture assay as well as an *in vivo* feeding trial to determine the effects of Original-XPC™ (XPC; a compound similar to a prebiotic) on *Salmonella* survival and cecal microbiota modulation. The *in vitro* study resulted in rapid and significant reduction (from approximately 1.0 to 2.0 logs) of the survival of *Salmonella* in the XPC treatment by the 24 h plating timepoint at both the 28 and 42 d sampling ages. However, by the 48 h plating timepoint at the 42 d sampling age, all the treatments containing cecal contents were able to reduce the *Salmonella* recovered to the same degree. These findings suggest the ability of XPC to accelerate the rate at which the cecal microbiota is able to reduce *Salmonella* invasion. Analysis of the cecal microbiota in both the *in vivo* and *in vitro* assays revealed no significant differences in species diversity and richness among treatments. However, cecal microbiota maturity revealed significantly increased species diversity and richness, indicating bird age to be critical in the modulation of the cecal microbiota.

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Dedication

Dedicated to my loving family and supportive friends

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Introduction

Antibiotic resistance among bacteria is an emerging issue within the United States that has been attributed to indiscriminate usage of antibiotics. As a result, antibiotic growth promoters (AGP) that were once commonly used in the food animal industry for improved growth performance and prevention of pathogen infection are now being prohibited from use to ensure a pure food supply (HHS, 2015). This retraction of AGP has proven to be a challenge for poultry producers in keeping their flocks free from infection, while maintaining the growth rates that were observed with the supplementation of AGP. *Salmonella* is one such pathogen commonly contaminating poultry. Therefore poultry producers and scientists support and conduct research to find and improve treatments that reduce and eliminate *Salmonella* observed in the poultry gastrointestinal tract (GIT). One treatment option is a product that modulates the intestinal microbiome of the host, allowing the GIT to be lined with a complex system of commensal bacteria: 1) stimulating pathogens to compete for space along the epithelial lining (Gabriel et al., 2006; Lawley and Walker, 2012), and 2) generating an unfavorable environment for pathogens, in turn prohibiting pathogen survival (Fooks and Gibson, 2002). This thesis begins with a comprehensive literature review (chapter one) that begins by describing the avian GIT and the GIT microbiome metabolic activities. The term "prebiotic-like compounds" is defined within this review for products that are similar to prebiotics and used in adjusting the GIT microbiome to maintain poultry health. The review concludes with a description of yeasts and their fermentation products, which act as a specific type of prebiotic-like compounds that are currently studied for their effects on poultry health. The remaining two chapters utilized a compound called Original-XPC^{TM} (XPC), consisting of a proprietary mixture of metabolites generated from the fermentation of *Saccharomyces cerevisiae.* This product and products similar

in nature have previously been reported to decrease *Salmonella* prevalence when supplemented to poultry diets, yet little is understood about its mechanism (Gao et al., 2008, 2009; Jensen et al., 2008; Osweiler et al., 2010). The second chapter of this thesis details a study using an anaerobic *in vitro* mixed culture assay applied to observe 1) the reduction of *Salmonella* survival and 2) the modulation of the microbiome while the cecal contents are supplemented with XPC. The third chapter uses a large-scale *in vivo* feeding trial to observe the effects that treatment with XPC has on the intestinal microbiome. This chapter allowed for the comparison of the less descriptive, yet rapid characterization method of a PCR- based denaturing gradient gel electrophoresis (DGGE) with the expressive and increasingly cost efficient method of next generation sequencing (NGS). Among the two chapters, it is important to note the credibility of the results from the *in vitro* assay as the comparison of those results to the *in vivo* feeding trial were revealed to be in agreement with one another.

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1. An introduction to the avian gut microbiota and the effects of yeast-based prebiotic-type compounds as potential feed additives

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Abstract

The poultry industry has been searching for a replacement for antibiotic growth promoters in poultry feed as public concerns over the use of antibiotics and the appearance of antibiotic resistance has become more intense. An ideal replacement would be feed amendments that could eliminate pathogens and disease while retaining economic value via improvements on body weight and feed conversion ratios. Establishing a healthy gut microbiota can have a positive impact on growth and development of both body weight and the immune system of poultry while reducing pathogen invasion and disease. The addition of prebiotics to poultry feed represents one such recognized way to establish a healthy gut microbiota. Prebiotics are feed additives, mainly in the form of specific types of carbohydrates that are indigestible to the host while serving as substrates to select beneficial bacteria and altering the gut microbiota. Beneficial bacteria in the ceca easily ferment commonly studied prebiotics, producing short-chain fatty acids, while pathogenic bacteria and the host are unable to digest their molecular bonds. Prebiotic-like substances are less commonly studied, but show promise in their effects on the prevention of pathogen colonization, improvements on the immune system, and host growth. Inclusion of yeast and yeast derivatives as probiotic and prebiotic-like substances, respectively, in animal feed has demonstrated positive associations with growth performance and modification of gut morphology. This review will aim to link together how such prebiotics and prebiotic-like substances function to influence the native and beneficial microorganisms that result in a diverse and well-developed gut microbiota.

Introduction

Poultry production in the past century has transitioned from predominantly breeding layers to breeding a mixture of both layers and broilers, based on the evolution of consumer demand (1–3). Success in the optimization of different broiler lines is due to genetics as well as optimizing diets with more precise nutritional formulations (4, 5). Comparison of individual genetic lines has revealed differing intestinal development, feed intake, and digestibility traits among other characteristics, which may impact performance (6–9). Improved diets have allowed broilers to reach their optimum body weight and feed conversion rate while minimizing mortality. Comparing poultry diets from the 1950s to those of the 1990s and 2000s illustrates the progress made (10, 11). For example, broiler chickens raised on a typical diet in 1957 had an average weight of 1,430 g at 84 days of age, whereas broilers fed a diet from 2001 yielded an average weight of 5,520 g at the same age. The feed conversion ratio in 2001 (2.68) was also considerably better compared to 1957 (3.26) (11). The current poultry diet contains the appropriate balance of amino acids, fatty acids, major and trace minerals, energy, and protein necessary for optimum growth (12).

Supplementation of various biologics have been attempted to enhance poultry feed for maximum growth development and health. Antibiotics enhance growth and reduce pathogens and although the exact mechanisms remain unclear, numerous working hypotheses have been offered (13–17). Antibiotic incorporation into poultry feed has since been tightly restricted and/or omitted due to microbial antibiotic resistance, presumably originating from both poultry (among other livestock) and humans (18–20). Since the exclusion of antibiotics in diets, a number of alternative supplements have been tried (Table 1.1), including prebiotics (21).

A prebiotic, as defined by Gibson and Roberfroid (35), is "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improves health." This definition has been subsequently refined to include the requirements for resistance to the acidic gastric environment, gastric enzymes, gastrointestinal absorption, and fermentation by the gastrointestinal microbiota while stimulating growth of beneficial intestinal bacteria (22). Being indigestible by the upper gastrointestinal tract (GIT) enables it to enter the lower GIT as a substrate for health-promoting bacteria, such as bifidobacteria and lactobacilli, thereby modulating the microbiota (35). Many feed additives currently used do not fit wholly into the strict prebiotic classification; they may lack one or more of the criteria set by Roberfroid (22). Although these substances have differing modes of action compared to prebiotics, they have a similar end result of a healthy and mature GIT microbiome. They may inhibit pathogenic invasion, reduce pathogens in the environment, modulate the host immune response, or enhance the host GIT morphology to enable the host to better limit pathogens in the GIT lumen. These substances will be referred to as prebiotic-like substances for the remainder of this review.

The objective of this review is to provide an overview of the effects of prebiotic-like substances, particularly those that are yeast-derived, while assessing the influence on microbial diversity of the poultry gut microbiota when using single or complex mixtures. In order to achieve this, both the gut microbiota as well as prebiotics is reviewed. Additionally, the characteristics of complex mixtures of prebiotic-like substances are assessed, including their effects on the gut development and physiology, the interactions that occur between host and microorganisms, and the potential use of prebiotic-like substances in creating a more healthy gut

microbiota. This review includes findings from not only poultry but also human and animal models, which may provide insight into potential effects in poultry.

Gut Microbiome: Terminology and Definitions

The microbiota is defined as the diverse population of microorganisms in a given environment, while the microbiome can be defined by either its genetic or ecological capacities (36). Genetic diversity is the entire collection of genes of the microorganisms in an environment, while the ecological diversity is all the microorganisms that make up an ecosystem (36). The term "microflora," once commonly used, is now often replaced by "microbiota" to avoid the plant connotation from the suffix "flora." (36). Regardless of the term used, it is essential to use a modifying adjective when referring to a specific anatomical region. For example, "gut microbiome" is indicating only the microorganisms in the GIT. There are numerous microbiome sites in addition to the gut microbiome, as they can be any shared anatomical sites between a community of microorganisms (commensal, pathogenic, or symbiotic) (37–39). An oral microbiome, for example, is the community of microorganisms that interact with and live within the oral cavity. It has several distinct microbial habitats within the oral cavity (gingival, tongue, and teeth) and extensions of the oral cavity (esophagus, middle ear, and nasal passages). Each different habitat within the oral cavity has its own distinct bacterial population in the form of complex biofilms (40). Research has shown that even the distinctive sites of the tongue – the dorsal and the lateral regions – possess differing bacterial profiles (41). Other frequently studied sites of microbiomes are the skin and the respiratory tract (42–44). The various regions and diversity among bacterial communities of the microbiota are indicative of the inherent complexity of microbiome research.

The gut microbiome is a widely studied topic because of its impact on health as well as its characteristic intricacy. The gut microbiome is home to one of the densest bacterial populations on earth, with numbers ranging from 10^8 to 10^{14}/g of digesta (45, 46). The microbiome encompasses biochemical and metabolic pathways not found in the host genome; this attests to the extent to which the microbiome has evolved (47). Microorganisms that comprise the gut microbiota have been found to directly impact the health of the host, providing protection against epithelial damage, aiding in digestion, and promoting development of a healthy immune system (48, 49). Commensal bacteria, in the GIT of animals, aid in absorption of nutrients as well as enhance nutrient utilization (50). Additionally, research conducted thus far has shown that earlier development of a mature and diversified microbiota leads to better growth of the host and fewer health issues, such as obesity, allergies, and asthma (51, 52). This is in part due to healthy competition among microorganisms.

Avian Gut Anatomy, Structure, and Functionality

For a thorough understanding of the microbial communities that inhabit the GIT of poultry and the effects they may have, a brief description of the poultry GI system is warranted. The GIT of poultry, chickens specifically, begins at the esophagus and continues down past the crop, proventriculus, and gizzard, through the intestines (duodenum, jejunum, ileum, and ceca), and ends at the colon and cloaca (53, 54). The gut microbiota generally refers to the intestinal regions and the studies included in this review focus on the duodenum, jejunum, ileum, ceca, and fecal contents as well as the structural characteristics to illustrate the gut microbiome of poultry. The ceca and their contents are most often studied based on their slow passage rate [comparatively, gut transit time from mouth to the lower ileum is approximately 3 h, while contents may be retained in the ceca as long as 35 h (55–57)] as it exhibits the most

diversification in the bacterial communities it harbors, in turn, indicating its impact on host health (54).

The intestines are multi-layered tubes, containing epithelial, muscular, and mucosal layers (58). Each section of the intestine, from the most proximal duodenum passing through the jejunum and out to the most distal ileum, contains numerous folds and is lined with villi and crypts. The villi are finger-like projections on the surface of the mucosal lining responsible for increasing surface area to maximize nutrient absorption and containing a meshwork of capillaries to allow nutrients entry into the bloodstream (59). When moving in the distal direction from the duodenum down toward the ileum, the mucosal lining reduces in thickness. The villi length and crypt depth also decrease in a continual gradation, which supports the notion of the majority of nutrient absorption occurring in the small intestine (58). Reduced intestinal weight is associated with improved nutrient absorption (60). Microscopic analysis has revealed that the reduction of intestinal weight is due to thinning of the epithelial lining rather than to the reduction in intestinal length, which is suggested to allow for improved nutrient absorption (61, 62).

The pancreas functions in hydrolysis of macromolecules, releasing digestive enzymes into the duodenum responsible for the hydrolysis of proteins, carbohydrates, and lipids supplied by the diet. In addition to enzyme production, the pancreas also produces hormones and bicarbonate that aid in metabolism regulation and buffer the intestinal pH, respectively (59, 63). The addition of enzymes to the duodenum allows for the small intestine to be the primary site of nutrient digestion and absorption. Having a general understanding of the digestive system of poultry allows for a more thorough insight into how microorganisms may impact GIT physiology. Turk (58) provides a more encompassing review of the entire avian GIT.

Avian Gut Microbiome Characterization

Characterization of microbial communities native to the poultry GIT began in 1901 and has since revealed these communities to be both diverse and dynamic (64). As biased culture-based methods advanced to molecular and sequencing techniques, a broader, more comprehensive representation of the microbiome has been recognized (64, 65). Researchers have attempted to determine a bacteriological profile of the poultry GIT via 16S rRNA gene-based studies; the findings have demonstrated that the majority of the 16S rRNA sequences in the cecal contents are not-yet-identified bacterial species (64, 66, 67). These discoveries uncovered the shortcomings of previously employed culture-based methods. For example, comparison of results obtained from Zhu et al. (64) and Rada et al. (68) found differing levels of *Bifidobacteria*-species present in untreated chicken cecal contents. Zhu et al. (64) used temporal temperature gradient gel electrophoresis followed by sequencing of the 16S rRNA fragments, while Rada et al. (68) used selective media; the experimental designs of both were comparable. The works of Zhu et al. (64) and Rada et al. (68) are two such examples for the characterization of the GIT microbiome; various techniques have been attempted to ascertain the microbial populations present in the different regions of the intestinal tract (Table 1.2).

Each area of the intestinal tract harbors distinct microbial communities. For example, the cecal contents exhibit greater levels of *Clostridiaceae*-related sequences as opposed to the ileum where more abundance of *Lactobacillus*-related sequences occurs (75). Apajalhti et al. (70) used G + C content to demonstrate similar results: the measurement of bacterial communities present in the ceca and ileum exhibited considerable variation when comparing the two G + C profiles. Variation in microbial communities is not only limited to differing organs, there is also a temporal factor in the nature of the microbiome (76). The cecal contents of younger birds appeared to possess more transient communities that matured into communities with much

greater complexity, while the ileum indicated an overall constant microbiome except at days 3 and 49 (the youngest and oldest sampling points) (75). The response to newly introduced microorganisms also appears to be dependent on sex of the host when analyzed in a mouse model; male and female GIT microbiota influence the metabolic activities and immune system differently (77). The concept of host factors affecting microbial diversity offers the opportunity to use established and healthy microbiomes to generate a working GIT microbial profile. However, this may prove to be quite challenging as it has been found that chickens interacting together in the same conditions, receiving the same feed, and of the same age and sex still display uniquely dominant bacterial communities (78). Although the exact quantities and qualities of a healthy microbiota have yet to be determined, a relationship appears to exist between the establishment of a mature intestinal microbiome and positive impacts on the host, resulting in improved growth and health (79).

Avian Gut Microbiome: Metabolic Activities

The poultry GIT is essentially coated in a dense layer of commensal bacteria in a diverse array of niches. Generally, the most complex microbial communities are found in the crop and the ceca. There is less colonization in the intestines based on the unfavorable environment. For example, the duodenum contains numerous enzymes, high concentrations of antimicrobial compounds, such as bile salts, and also has a rapidly changing environment due to reflux from the jejunum up to the gizzard (80). Traveling further down the GIT, the ileum and ceca become more favorable environments with fewer enzymes and antimicrobial compounds; this is reflected in the increased concentrations of commensal bacteria, around 10^9 and 10^{11} bacteria/g, respectively (46). The unique anatomical structure of the cecum allows for the occupancy of fermentable substrates not widely available in different areas of the GIT; this enables differing

microorganisms to reside and produce large amounts of energy metabolites to aid in achieving the bird's energy requirements (81).

Research profiling whole body energy consumption patterns has attributed 22.8% to being utilized by the GIT and liver (82), but not all of that energy is actually being used by and for the host. It was reported that the presence of GIT microbiota significantly increased the dietary metabolizable energy in the broiler chicken host, indicating that the microbiota are responsible for utilizing the additional dietary energy (83). The commensal bacterial communities utilize nutrients from the host's diet as energy sources, making those nutrients unavailable to the host. However, they are able to produce short-chain fatty acids (SCFAs) from the fermentation of those nutrients (84). Research suggests the GIT microbiota aid in digestion and energy release from starch and fibrous contents, especially in the ceca. It is proposed that the amounts and types of SCFAs that are generated in the ceca are in proportion to differing starches that enter the ceca (85). Although SCFAs serve as additional energy sources for the host, it is suggested that only a proportion (up to 25%) of the overall SCFA energy is recovered by the bird (85, 86). In high-fiber and low-energy diets, bacterial digestion of the fiber also releases energy in the form of SCFA (84, 87). Along with generating accessible energy, the gut microbiome is associated with conservation of energy when nutrient sources (proteins, fats, and sugars) are low (88, 89). The production and absorption of SCFAs in the intestine are occurring continuously, with more or less being produced due to alterations in the diet or cecal microbiome (85).

Conversely, the resident microbiome has also been associated with unfavorable effects to the host's utilization of dietary energy. Although the presence of the GIT microbiota has indicated a significant increase in levels of metabolizable energy in conventionally raised broiler chickens when compared to germfree (89), the metabolizable energy is attributed to the products

generated by the GIT microbiota. The variation can be associated with the digestibility of those energy sources (dietary fiber and starches) being broken down into monosaccharides and SCFA. The SCFA are portrayed as possessing a high metabolic energy value, yet they are inefficiently utilized by the host. Therefore the levels of SCFA present are not reflective of the net deposition of energy to the host (86, 89). Another potential explanation may be that the presence of the gut microbiota increases the cost of energy by altering the rate of energy-consuming reactions (89, 90). For example, pathogens attach to the epithelial lining, alter its integrity and function, and in turn stimulate the renewal of epithelial lining, which increases the amount of dietary energy spent on gut maintenance (27, 91). It has also been observed that conventionally raised birds have higher energy requirements for maintenance when compared to germfree birds (92). This may be due to the addition of the host's microbiota usage of metabolizable energy, or the host's microbiota making dietary energy unavailable to the host (92).

Avian Microbiome and Foodborne Pathogens

The complex lining of the lower intestines with bacteria serves as a barrier against colonization of pathogenic bacteria, which if allowed to occur, could lead to infection. The bacteria that settle first in the lining of the intestines necessitate that any other microorganisms in search of new residence must compete for space and nutrients in order to survive and colonize (80, 93, 94). Establishing the early foundation of a mature GIT microbiota has been associated with prevention of infection with pathogens, namely *Salmonella*, by beneficial bacteria outcompeting the pathogenic bacteria for space and nutrients (95–98). In nature, chicks are hatched in the presence of maternal fecal contents, allowing rapid colonization of members from the maternal gut microbiome (25). In an attempt to colonize newly hatched chicks with a mature and healthy microbiome that will discourage pathogenic bacteria from colonizing, chicks have

been experimentally inoculated with competitive exclusion culture mixtures (97, 99–102). Introduction of the competitive exclusion cultures has proven to be effective in protecting young chicks from enteric pathogens and several reviews have been written on various aspects of this research (103–106).

As previously mentioned, commensal bacteria produce SCFA, which are recognized as having growth-inhibiting effects on enterobacteriaceae (107–109). The presence of the SCFA causes a drop in cytoplasmic pH, which is recognized as a contributing factor to the inhibition of pathogen growth (110). Although the mechanisms of SCFAs are not well understood, they are known to exhibit bactericidal and bacteriostatic properties (30–32, 111). Russell (30) suggested that it is not only the result of a drop in pH caused by the SCFA but also the uncoupling reactions produced by the translocation of protons by SCFA that contribute to the growth inhibition effects seen. In accordance with this notion, Davidson et al. (112) suggested that because the fatty acids produced are weak acids, they are effective as antimicrobials in their undissociated forms as they are able to easily diffuse through the cytoplasmic membrane of the microorganism. The fatty acids dissociate into anions and protons once in the cytoplasm of the microorganism (maintained relatively neutral or slightly alkaline), in turn decreasing the pH and causing conformational changes of cytoplasmic proteins, enzymes, and nucleic acids. In an attempt to reestablish a neutral/slightly alkaline pH, microorganisms utilize ATP-dependent pump systems to transport the anions and protons outside of the cell. This is in accordance with findings of Cherrington et al. (113), where incubation of *Escherichia coli* with propionic and formic acids resulted in reduced rates of macromolecular synthesis initially, yet it partially regained synthesis rates after continued incubation.

Anion accumulation is suspected to be another factor in uncoupling reactions that attributes to growth inhibition of bacteria in the presence of SCFA. It is suggested that the accumulation of acid anions causes an uncoupling effect of the electron transport chain from oxidative respiration (via the passage of molecules in their dissociated and undissociated forms, transferring protons into the cell to dissipate the proton motive force) as well as a chaotropic effect (disrupting hydrogen bonding in water causing macromolecules in solution to lose stability) that are accountable for the increased hydrogen ion leakage into the cell. The cell is unable to excrete hydrogen ions rapidly enough, making it difficult for the cell to regain its neutral/slightly alkaline intracellular pH (30, 110, 114, 115). The intracellular increase in hydrogen is unable to counteract the accumulation of acid anions (116). Another inhibitor of bacterial growth by SCFA is the disruption of the membrane of a microorganism by means of permeabilization or intercalation, allowing for the release of macromolecules and the destabilization of the membrane (117, 118). However, there are instances of pathogenic bacteria acquiring resistance to SCFAs (32). For example, pre-incubation of *Salmonella* with high concentrations of SCFA at neutral pH resulted in an acid tolerance response and has also been demonstrated to be responsible for modulation of virulence gene expression and attachment/ invasion of *in vitro* tissue culture cells (119–122).

While the production of fatty acids is inhibitory to invading bacteria, studies suggested that the fatty acids are inactive against the species that produced them (123). Smulders et al. (124) found that acid-producing bacteria are tolerant to acids and in turn, the acidic environments that they generate. Therefore, the influences of the SCFAs produced by autochthonous bacteria may provide protection against pathogenic bacteria – *Salmonella*, coliforms, and *Campylobacter* – intent on colonizing in the intestine while leaving commensal bacteria

unscathed (125). However, little else has been reported on the effects of the fatty acids on the producing species.

Key Players in the Gut Microbiota

In the past, the microorganisms colonizing the GIT were thought to be commensal, neither beneficial nor harmful to the host, as opposed to being mutualistic (37). However, numerous germfree experiments in various animal models have indicated the value of these indigenous microorganisms (126–128). There has been overwhelming data collected revealing the beneficial impacts on both host physiology as well as immunology (75, 129). Several studies have indicated that introducing a balance of beneficial microorganisms to the poultry microbiota improves body weight gain and feed conversion ratio as well as warding off common diseases in poultry, such as Newcastle disease and infectious bursal disease (130–132). However, in order to better promote strategies for increasing the presence of beneficial bacteria, those bacteria and their interacting counterparts must be identified.

Although being incredibly diverse, the most abundant microorganisms in the gut microbiota of poultry are primarily anaerobic (54). This is somewhat expected since there is little to no oxygen available as an electron acceptor in the lumen, although the concentration of oxygen is greater toward the epithelium, thus forcing bacteria to use fermentation to produce pockets of organic acids within the lumen (133). Moreover, Sun and O'Riordan (133) suggest that as a result of this environment, it is necessary to investigate SCFAs more in depth because bulk analysis does not reveal the true nature and spatial arrangement of these acids (which would further indicate the location and family of anaerobic bacteria). There is no consistent data available indicating the overall Gram status of poultry GIT microbiota. Investigation into the commensal bacteria present in an untreated chicken ceca has resulted in an array of bacterial

communities (Gram-positive Y-branched, Gram-positive non-sporulating, Gram-negative) and may be attributed to the rearing conditions, chicken breed, diet, or even the cultivation and enumeration methods applied for bacterial characterization (125). Nevertheless, there are trends observed in available data investigating the microbial populations in broiler chickens grown in a conventional poultry flock and those raised under laboratory conditions (76, 134).

Lactobacilli and bifidobacteria are two of the more well-known beneficial bacteria, however, there are numerous others: *Bacillus*, *Enterococcus*, *E. coli*, *Lactococcus*, *Streptococcus* as well as undefined mixed cultures (Table 1.3) (23). These bacteria are indigenous to the GIT, occupy space, and consume nutrients along the intestinal tract, limiting the colonization of pathogenic bacteria. In addition to competing for space and nutrients, these bacteria have been recognized for exporting bacteriocins, which can target and kill invading pathogens (133). All of these microorganisms fit under the umbrella term probiotics. Like prebiotics, probiotics also have specified criteria and characteristics: (1) non-pathogenic and of host origin, (2) resistant to gastric pH and processing/storage, allowing them to persist in the intestinal tract, (3) able to adhere to epithelial and mucosal membranes, (4) modulate immune responses, and (5) produce inhibitory compounds (23). It is the complexity and broad diversity of the beneficial microorganisms that make up the microbiome and allow for a mature and healthy host (51, 52).

Bacteria may be beneficial to the host by aiding in degradation of polysaccharides otherwise indigestible to the host. The monosaccharides produced can be subsequently broken down further into SCFAs and lactic acid (37). As previously mentioned, both lactobacilli and bifidobacteria are beneficial and indigenous to the human and chicken GIT (145). Lactobacilli are members of a group collectively referred to as lactic acid bacteria, which metabolize

carbohydrates to produce lactic acid as the primary end product (146). Oligosaccharides are their main nutritional source, which is reflected in their residence in ecological niches rich in carbohydrate-containing substrates, most commonly plant material, spoiled or fermented foodstuffs, and mucosal membranes of humans and animals (147). Along with their broad range of habitats, lactobacilli are able to adapt to various conditions by altering their strictly fermentative metabolism accordingly; they may be obligately homofermentative, facultatively homofermentative, or obligately heterofermentative (148). Their fermentative status is based on the levels and proportions of end products they generate from fermentation of differing substrates (although other factors, such as chemical and physical environment, play a role in determining fermentative status). Obligately homofermentative indicates that their primary fermentation product is lactic acid (>85%) generated by fermenting hexoses (149). Facultatively homofermentative indicates that they are capable of fermenting hexoses and pentoses using different pathways to generate lactic acid (although under low substrate concentration and strictly anaerobic conditions, they are capable of producing acetic acid, ethanol, and formic acid). Obligately heterofermentative lactobacilli ferment hexoses generating equimolar amounts of lactic acid, CO_2, and acetic acid (148–150). Although the end products produced are a fair indication of fermentative status, they are not the sole factor. These microorganisms are aerotolerant and acidophilic, allowing for the GIT to be an optimal residence (146, 151).

Bifidobacteria are another well-documented example of beneficial bacteria. They are often associated with lactic acid bacteria for their production of lactic acid, however, they are phylogenetically distinct. Bifidobacteria are Gram-positive, heterofermentative, and non-motile (152). Like lactobacilli, bifidobacteria digest oligosaccharides to use as carbon and energy sources, to produce lactic acid, acetic acid, ethanol, and formic acid (153). They are not

exclusive to the utilization of dietary compounds, they can also digest carbohydrates produced by other members of the GIT (154). Additionally, they are capable of internalizing simple sugars remaining in the environment, thus preventing pathogenic bacteria from utilizing them as a nutrient source (155).

Both lactobacilli and bifidobacteria are known to be members of the intestinal microbiota in animals and humans; their presence is important for the maintenance of the GIT microbiota (156–158). Being that lactobacilli and bifidobacteria are autochthonous and dominant in the GIT, they can be utilized as a control method of pathogenic bacteria by competition, for example *Clostridium perfringens* (156). Lactobacilli and bifidobacteria possess characteristics that allow them to out-compete pathogenic bacteria. Various strains of lactobacilli adhere to intestinal epithelial-like cells and exhibit antimicrobial activity against bacteria typically found in the (human) GIT (157). A link between the lactobacillus strain's pH tolerance and antimicrobial properties has been reported, both *in vitro* and *in vivo* (157).

Different species of lactobacilli and bifidobacteria produce various antimicrobial agents, which allow them to be inhibitory toward pathogenic bacteria. Many species of lactobacilli and bifidobacteria produce SCFA; the production of these acids causes a drop in intestinal pH. The lowered pH level extends the lag phase for sensitive microorganisms (124). The undissociated forms of these acids are able to penetrate the microbial cell and hinder metabolic functions (further information on the mechanisms of these acids was discussed in a previous section of the current review). Another end product generated from lactobacilli and bifidobacteria is CO_2, which has demonstrated inhibition of microbial growth (149). The inhibitory mechanism of CO_2 is unclear, although Eklund (159) was able to rule out the proposed mechanism of CO_2 accumulation in the membrane of the microorganisms, physically interrupting the bacterial

membrane. Growth of *E. coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Bacillus cereus* has been shown to be inhibited in the presence of CO_2 at various concentrations (159). Succinic acid is produced by both lactobacilli and bifidobacteria, although at minimal levels (160, 161), and is associated with antibacterial activities in a multitude of environments (162, 163). Diacetyl is an end product of lactobacilli that exhibits antimicrobial effects. It is suggested that diacetyl is more effective in a lower pH (≤7) causing it to be lethal to Gram-negative bacteria and inhibitory of yeasts (164). Bacteriocins, produced by lactobacilli, may have a narrow or broad range of activity. Lindegren and Dobrogosz (149) have reviewed the various antimicrobial agents produced by lactic acid bacteria in more detail.

Overgrowth of any single type of bacteria can have unfavorable effects on the host. Lactobacilli are considered beneficial bacteria, however, antibiotic growth promoters that stimulate improved growth of broilers were also associated with heightened sensitivity of lactobacilli to those antibiotics (165). Although the host may benefit from the commensal bacteria competing with pathogenic bacteria, an overgrowth of commensal bacteria can be detrimental to the host by excessive uptake of nutrients making them unavailable to the host (166). Additionally, overgrowth of lactobacilli can impair host fat absorption by not allowing proper biotransformation – deconjugation and dehydroxylation – of bile acids (14). Overgrowth of bacteria can also lead to overproduction of fermentation end products to the detriment of the host. For example, overgrowth of *Streptococcus bovis*, a commensal lactic acid-producing bacteria can generate considerable acid production and a concomitant lowering of the surrounding environment pH. This sequence can be advantageous for competing against pathogens. Consequently, under *in vitro* incubation conditions in co-culture with *Salmonella typhirmurium* growth of *S. bovis* can behave as a probiotic and directly limit *Salmonella* growth

as a function of carbon source and time of inoculation (144). However, when easily fermented carbohydrates are fed to ruminants, excessive *S. bovis* growth can occur in the rumen resulting in rapid lactic acid overproduction, subsequent lowering of the ruminal pH, and the eventual development of a harmful ruminal lactic acidosis condition in the animal (167). Therefore, even though *S. bovis* might be considered a gut commensal organism, and in some cases a probiotic candidate, it can also be associated with host clinical disease states, such as bacterial endocarditis and colon cancer in humans (144).

Introduction and History of Prebiotics

The most widely accepted definition of prebiotics are non-digestible feed ingredients that are selectively fermented by beneficial bacteria in the lower GIT (capable of withstanding harsh conditions in the upper GIT) so as to provide energy to promote bacterial growth and metabolism in the colon which contributes to specific changes that lead to improved host health (22, 35, 168). Colonic food is a non-digestible ingredient that makes it past the upper GIT and into the colon, serving as a substrate for non-specific bacterial inhabitants, both beneficial and harmful (169, 170). Not all colonic foods are necessarily prebiotics; the rationale for designating a compound as a prebiotic or not depends upon whether beneficial bacteria alone are able to digest it. Some miscellaneous compounds that serve as colonic food, but do not fall into the category of prebiotics because of the non-specific targeting of microbiome bacteria include resistant starch, non-starch polysaccharides, non-digestible oligosaccharides, and yeast fermentation products (171). There have been numerous studies conducted and reviews written covering common prebiotics and their beneficial impacts; therefore they will not be discussed in detail here (Table 1.4) (35, 172–174).

Some lesser-studied prebiotic-like compounds are *Saccharomyces cerevisiae* fermentation products (SCFPs) or yeast culture (YC) components; these compounds do not fall into the precise definition of prebiotics as set by Roberfroid (22), among other classical definitions. However, they have prebiotic-like effects in that they have been shown to enhance nutrient utilization and digestibility, as well as improving the immune system and inhibiting pathogen-intestinal cell interaction by modifying the GIT microbiome (179–181). The fermentation of *S. cerevisiae* –undefined strains – produces SCFP. They include the fermentation products and metabolites, the media used in the fermentation to preserve fermentation activity, and both the yeast cell wall fragments and residual live yeast cells; thus, they share characteristics in both probiotic and prebiotic realms (179). There are commercial YC products available that are being more thoroughly investigated to identify their exact effects and maximize the directed influence(s) they may have.

Because yeasts are most often associated with the wine making, brewing, baking, and other fermenting industries, it is critical to consider why these unique organisms were initially promoted for use in improvements of animal and human health. In order to do this, a brief review of the history of yeast that led to its usage as a feed additive is discussed in the following section.

Introduction to Yeast: History and Background

To understand the current use of yeast and yeast products in food and agricultural settings, it is important to at least briefly describe the history of yeast in scientific applications and the evidence for the close relationship among yeast strains originally uncovered and those used in today's laboratory-based research. Humans began using yeast over 7,000 years ago, with its earliest usage dating back to the Neolithic times for wine making (182, 183). In the past century, yeasts have been investigated on a genetic level after the Carlsberg Laboratory

introduced scientific concepts to the brewing industry, as discussed by Greig and Leu (184). In the 1930s, the genetic analysis of yeast became accepted based on its potential as an experimental organism; it was pioneered by Øjvind Winge and Carl Lindegren (185). Winge used a strain isolated from the Carlsberg Laboratory, while Lindegren used a strain, EM93, isolated from rotting Californian figs (182). Yeast continued gaining popularity in the scientific field for its ease in gene manipulation (182). In the 1950s, Robert Mortimer constructed the strain S288C, which has been purported to share more than 85% of its genome with EM93, Lindegren's original strain (most laboratories involved in the analysis of yeast use a derivative of EM93 – a strain of *S. cerevisiae*). This strain was subsequently sequenced in 1996, making it the first fully sequenced yeast genome (186, 187). For further purposes of the current review, *S. cerevisiae* is the main species of yeast discussed unless otherwise indicated.

Yeast in the Laboratory

A renowned model organism, yeast is a single-celled fungal eukaryote that most often divides by budding. Yeasts are used in various industries because of their ability to ferment sugars in the absence of oxygen to produce CO_2 and alcohol. In a laboratory setting, yeasts are most often used for analysis as a model template to study higher eukaryotic organisms. Yeasts are ideal for studying processes known to occur in more complex eukaryotic organisms because even though yeasts are unicellular, they encode similar proteins and are thus representative of more complex organisms at the cellular level (188). When comparing all yeast protein sequences to mammalian sequences, of the potential protein encoding regions in yeast, "statistically robust" homology among the two was observed (189). Because of the lack of mammalian protein families and proteins sequenced, there may be much greater similarities between the two.

Part of the attraction of yeast as an experimental model is the ability to easily manipulate and mutate genes, either on plasmids or in the yeast chromosome itself, to view the resulting phenotypic effects (182). An insight into its fairly simple manipulation is evident in research performed by both Caspeta et al. (190) and Liu et al. (191). Caspeta et al. (190) manipulated *S. cerevisiae* into expressing thermotolerance to temperatures ≥34°C (typical response to these temperatures is serious impairment of function) by exposing the isolate for short stretches of time to increased heat followed by serial batch transfers. This resulted in non-inheritable heat tolerant strains that exhibited increased growth rates as well as increased glucose consumption rates at higher temperatures when compared to thermolabile strains (190). Thermotolerance has also been bestowed upon *S. cerevisiae* by the introduction of genes from organisms that are naturally thermotolerant. This transfer of genes allows for inheritable alteration in future generations of *S. cerevisiae*. Duina et al. (182) illustrated the extent to which yeast has proven its efficacy as a model organism, discussing research advancements and accolades (Nobel Prize and Lasker Award) in an array of fields achieved by utilizing yeast.

Although great progress has resulted from the study of yeast, it has also stimulated further inquiry. Yeast researchers began with the goal of determining functions of single genes and proteins, but now seek a "systems level" approach. The benefit of understanding how proteins interact to maintain cellular functions (metabolism, reproduction, growth, regulation, signaling, and homeostasis) is now at the forefront for yeast biology (192). Yeast's position as a model organism for various scientific fields is reviewed more thoroughly in several articles and therefore will not be further discussed here (192–194). A review by Siddiqui et al. (195) encompasses the potential of engineering yeasts to contain secondary metabolite pathways for pharmacological purposes. Additionally, Sherman (196) has generated a comprehensive review

(both extended and truncated versions available) on the biological basics of yeast, which includes a section on a variety of outside literature references for yeast.

Yeast Metabolism

Yeasts are capable of cellular respiration in the presence and absence of oxygen; for this review, we will discuss respiration only in the absence of oxygen, as it is most applicable to the topic of the current review. Anaerobic respiration, or fermentation, is the process of breaking down sugars to generate energy for carrying out cellular processes. In anaerobic cellular respiration, sugars are broken down into pyruvate and subsequently decarboxylated and reduced to form CO_2 and ethanol. For fermentation to begin, any complex sugars must be broken down into simple sugars (e.g., sucrose to glucose and fructose) via enzymes from yeasts, adding an additional step to the fermentation process (197). In the process of understanding this, it is recognized that complex carbohydrates (starches and fiber) are more challenging for yeasts to ferment than simple sugars. Investigation into the types of sugars and environments yeasts are capable of fermenting is necessary to optimize the production and utilization of yeast fermentation products. By understanding the conditions in which yeast fermentation is optimized, they can be engineered to generate additional metabolites that may prove to be beneficial for use in animal feed.

Yeast as an Animal Feed Additive

The usage of live yeast and yeast products in animal feed is not a new concept, although pinpointing the exact point of its conception has proven to be challenging. It is suggested that the introduction of YC in animal feed was not until the 1980s (198). It appears that the majority of research has been dedicated toward ruminants, while equine, porcine, poultry, and companion animals received attention to a lesser extent. Initially, yeast was used in an array of modes

because of the large quantities of yeast biomass waste generated by distilleries (and other yeast utilizing industries) (199). It was used as a feed additive because it was a rich source of protein, fiber, and minerals. It has been hypothesized that both viable and non-viable yeast cells provide essential B vitamins and organic acids (200). In the past, both viable and non-viable yeast cells have been added to animal feed – including poultry feed – and resulted in increased host growth and improved health (199).

It is essential to have a precise definition for YC, so it is not confused with using live yeast (probiotic/direct fed microbial form) or yeast extract (only soluble portion of yeast autolysis) products (201). As described in a previous section of the current review, YC contains the cellular constituents as well as residual viable cells. It is effective when used because it contains lysed yeast cells; this allows for the nutrients within the yeast cells to be available for digestion and absorption (202). These yeast cells are lysed by autolysis; they are subjected to temperature or osmotic shock, thereby killing the yeast cell while leaving the endogenous enzymes undamaged. The yeast cell's own enzymes begin to degrade the yeast cell, releasing its contents and further degrading its proteins into amino acids (203). Some yeast cells that are capable of tolerating the temperature or osmotic shock, do not autolyze, and remain metabolically active.

The mode of action of YC is seen to enhance digestive and fermentative functions of the GIT, while modifying activities of the GIT microbiota, although the mechanisms are less clear (198). Based on *in vitro* and *in vivo* studies, supplemented YCs appear to have several impacts on the rumen microbiota including increased numbers of beneficial bacteria and fiber digesting bacteria as well as shifting away from hydrogen consuming methanogens and toward bacteria capable of converting hydrogen and CO_2 to acetic acid, all of which could, in turn, potentially

benefit the ruminant host animal either directly or indirectly (204, 205). Enhanced growth performance resulting from the supplementation of YC with probiotics (*Lactobacillus acidophilus* and *Streptococcus faecium*) has indicated its potential effect of increasing digestion and absorption of the GIT microbiota occurring in broiler chickens (206). de Oliva Neto et al. (207) conducted studies on the antibacterial properties of YC supernatant, which indicated a reduction of pathogenic bacterial growth when tested against a common distillery bacterial species. Interestingly, the supernatants were tested as both fresh and post freeze/thaw, and reported similar results indicating the antimicrobial activity could withstand freezing. Conversely, when heat (90°C for 20 min) was applied, the antibacterial activity was destroyed. Accordingly, YC and yeast extract have yielded varying results, which suggests the necessity for metabolically active yeast cells. When supplementing heat-treated inactive yeast cells to steer diets, there was no effect on the concentrations of cellulolytic bacteria, while supplementing live, metabolically active yeast cells increased the concentration of cellulolytic bacteria (208).

In addition to their ability to interfere with bacteria due to their relative large size, supplementation with live yeast products has led to a few suggested modes of action (209). One mechanism suggested by Jouany et al. (204) involves metabolic competition with bacteria that may be adhering to and digesting fiber or starch molecules. In this scenario, the yeasts ferment the carbohydrates produced, prohibiting their usage by other bacteria. Another mechanism of action of live yeast cells is their ability to produce protective products with antitoxin effects (210). Yeast intake has resulted in a stimulation of activity of host intestinal brush border enzymes, which has counteractive effects to those of pathogens, along with supplying the host with additional enzymes (211). Elimination of oxygen has been deemed the most influential mode of action in ruminants (212). Although there is little oxygen present in the GIT, live yeast

cells scavenge for excess oxygen introduced by food and water intake; this allows for a more optimal environment for anaerobic bacteria (204, 212). Most all implications regarding the mechanism of oxygen elimination have been derived from studies conducted on ruminants.

As noted previously, the majority of the studies on the effects and mechanisms of YC have been performed on ruminants. Although such studies may be a good indicator of the potential use of YC in other animals, it can also be expected that there will be differences seen among ruminants and non-ruminants. For example, considerable research has been conducted on the effects of milk production in cattle, while this is beneficial for other lactating animals, the information gleaned from these studies holds little merit for poultry researchers. Instead, conducting *in vitro* and *in vivo* studies on specific animal subjects of interest would be more useful in identifying the mechanisms of YC in those animals rather than projecting ruminant/rumen microbiota results onto non-ruminant species.

Impact of YC on Host: Microorganism Interactions

The effects of YC on the intestinal morphology in swine have indicated increased jejunal villi width, which allows for greater digestive and absorptive intestinal capacity leading to better body weight gain when compared to controls (180). In contrast, poultry data obtained has thus far indicated significant differences in intestinal morphology (213–215). Supplementation of YC has resulted in more shallow crypt depths, indicating less necessity for cell renewal and turn-over, allowing for decreased host energy utilization for intestinal epithelial maintenance (216). Feed efficiency and body weight gain have both resulted in significant increases when YC, yeast derivatives, and live yeast cells are added to the poultry diet (215, 217, 218).

Inclusion of YC in animal feed has led to suggestions that they may aid in the clearing of pathogens from infected animals. A study involving the inoculation of pigs with *Salmonella*

suggested that the inclusion of YC in the diet allowed for rapid shedding of the pathogen from the GIT (180). Supplementation of broiler feed with YC has also been seen to enhance adaptive immune system T lymphocytes, allowing for better clearing of the pathogens (181). El-Husseiny et al. (219) observed that commercial YC were able to significantly increase antibody production against SRBC, much in agreement with the findings of Al-Homidan and Fahmy (220), who reported significantly higher antibody titer concentrations in response to Newcastle disease in broilers fed YC.

Further examination into the components of yeasts' cell walls indicates the beneficial structural polysaccharides present and released into culture when yeast cells autolyze. Mannan-oligosaccharide (MOS) is included in the YC as it is derived from the outer cell wall of *S. cerevisiae*. MOSs bind to pathogenic bacteria in the GIT, preventing their attachment to the mannan residues on intestinal epithelia (221). This not only protects the host from pathogens but also allows for host energy reserves to be utilized for their own growth rather than to the repair and regeneration of the epithelial lining (222). β-glucans are also released when the yeast cell wall is degraded; presence of these molecules can lead to pathogen inhibition along with immuno-modulating effects. Similar to MOS, β-glucans act by preventing pathogens from binding to the villi of the gut mucosa (214, 216). Additionally, β-glucans are known to activate phagocytes, natural killer cells and B and T lymphocytes as well as increase cytokine production and phagocytic activity of macrophages (223).

Mannan-oligosaccharide supplementation has been reported to increase broiler growth performance when supplemented in their diet (224, 225). *In vitro* experimentation has indicated that addition of MOS inhibits the attachment of enteropathogenic *E. coli* to the gut mucosa as well as removing attached *E. coli* from the mucosa (226). Inclusion of yeast fermentation

products, like MOS, appears to reduce pathogenic bacterial populations. The mechanism is unclear, although the agglutination of the pathogens with sugars from the yeast cell wall occurs rather than attachment to the host intestinal lining is one hypothesized mechanism (227). Yang et al. (228) indicated MOS altered the gut microbiota of broilers and reduced the number of mucosal-associated coliforms.

Although some studies suggest a positive association between yeast and growth promotion (229, 230), other studies have indicated no positive effects on inclusion of YC in broiler diets (231). Paryad and Mahmoudi (229) indicated that inclusion of 2% yeast (*Saccharomyces cerevisae*) in broiler chicken diets resulted in significant differences in body weight gain, feed intake, and feed conversion rate when compared to controls. Similarly, investigation into YC on growth promotion in lambs suggested its efficacy, resulting in increased feed intake and growth by 8 and 26%, respectively. Conversely, similar research conducted on lambs evaluating the efficacy of three yeast strains and a mixed culture resulted in little consistency and lacked an overall effect when compared among yeast strains (232). Adebiyi et al. (231) also showed no significant differences in body weight gain in broiler chickens when fed varying percentages of YC.

Yeast Metabolites and Metabolism as Prebiotic-Like Substances

In addition to the structural polysaccharides derived from the yeast cell wall, yeasts generate a number of metabolites that may offer benefits to the host animal when supplemented to animal feed. Metabolites include carotenoids, vitamins, enzymes, amino acids, and some miscellaneous products (200). Several yeast species are naturally capable of producing carotenoids (including β-carotenes), which are subsequently metabolized into vitamin A (200). Vitamin A aids in cellular differentiation and proliferation, making it critical for intestinal

maintenance and health (233). The enzyme responsible for the synthesis of vitamin A from β-carotene is β,β-carotene 15,15′-monooxygenase, which has been isolated and characterized from the intestines of poultry, among other animals (234, 235). Although *S. cerevisiae* is not capable of naturally producing carotenoids, it is capable of and has been engineered to express a biosynthetic pathway for the production of β-carotene (236).

Other vitamins (vitamin precursors) produced by yeasts include ergosterol, L-ascorbic acid, and D-erythroascorbic acid. Ergosterol is particularly abundant in *S. cerevisiae*, accounting for up to 90% of the total sterols (237). It is located in the membrane of yeasts and is responsible for its fluidity, structure, permeability, and activity of membrane-bound enzymes (238). Ergosterol is a precursor to both vitamin D_2 and cortisone (239). Vitamin D_2 is responsible for the proper absorption and transport of calcium, among other minerals (240). D-Erythroascorbic acid is also synthesized by *S. cerevisiae* and depending on the substrates available, that pathway can be manipulated into producing L-ascorbic acid (vitamin C) (241). The ingestion of vitamin C has been suggested to alleviate some of the repercussions of heat stress: poor immune function and growth performance (242). However, instances of supplementation of L-ascorbic acid in poultry diets have had varying results; some resulted in increased levels of superoxide dismutase in 45-week-old broilers, while others revealed no effect on the activities of antioxidative enzymes, superoxide dismutase included in 7-week-old broilers (243, 244).

Yeasts are recognized for their production of enzymes expressing various activities (245). Jones (246) wrote a comprehensive review documenting the activities of the proteolytic systems in *S. cerevisiae*, along with mentioning other enzymes elucidated in *S. cerevisiae* (carboxypeptidases, aminopeptidases, and dipeptidyl aminopeptidases). An enzyme in *Saccharomyces boulardii*, a subtype of *S. cerevisiae*, was found to degrade the ileal receptors

in rats for toxin A generated from *Clostridium difficile* (a food-associated pathogen causing gastroenteritis; one study isolated *C. difficile* from 2.3% of broiler chickens tested) (247, 248). The degradation of the receptors prohibits the toxin from binding and prevents infection from occurring (249, 250). There have been multiple other proposed mechanisms of action for yeast on the immunoprotective effect in the GIT, specifically the prevention of *C. difficile* infection: (1) *S. boulardii* releases proteases that hydrolyze toxins and prevent its binding to the intestinal receptor (250), (2) *S. boulardii* is capable of stimulating the activity of disaccharidases in the intestinal brush border with no additional alterations of the intestinal mucosa (211), and (3) *S. boulardii* increased the production and secretion of glycoproteins, namely the secretory component of immunoglobulin A (251). Potentially, by narrowing the focus on the exact mechanism of action, *S. cerevisiae* could be engineered to confer said mechanism and supplemented into animal (poultry) feed to prevent colonization of *C. difficile*.

Invertase is another enzyme produced by *S. cerevisiae*; it hydrolyzes sucrose into glucose and fructose (252). Invertase efficiency and sucrose availability allows for glucose to be a carbon source for *S. cerevisiae* (252). Ideally, provided the diet contained appropriate levels of sucrose, one could engineer *S. cerevisiae* to overproduce invertase and subsequently add it to poultry feed. This would allow increased production of glucose, available not only for its own needs but also for other microorganisms in the surrounding environment. This mode of action would not be selective toward beneficial bacteria in the microbiome.

Yeasts have multiple amino acid transport systems; amino acids are incorporated into proteins or they are broken down and utilized as nitrogen and carbon sources to promote growth (253). Yeasts and yeast derivatives are capable of producing amino acids; therefore supplementation to animal feed would provide both the host and the microbiome with amino

acids. Almquist (254) reviewed the essential amino acid requirements in young chicks, laying hens, and turkeys; Almquist included a table outlining the percentages of each amino acid to reach a specific protein level. Amino acids are necessary for poultry to have proper growth and promote efficient weight gain and feed conversion ratios (255). Lysine appears to be one such amino acid that plays a significant role in the body composition of poultry (256). Mutants of *S. cerevisiae* have been revealed to produce up to 17 times as much lysine as wildtype; thus this rich source of lysine may prove to be valuable to the growth and development of poultry (257).

Miscellaneous metabolites are also produced in *S. cerevisiae*, including toxins responsible for the "killer phenomenon." Originally, this phenomenon was considered to be lethal only toward members of the same species; however, further investigation has led to the recognition of these toxic species to have destructive consequences reaching both prokaryotic and eukaryotic organisms (258–261). Polonelli and Morace (261) acknowledge that the inhibition of outside species may not be a direct impact on the toxins secreted, but more of a concerted effort from multiple metabolites. Nevertheless, these toxic species of *S. cerevisiae* are displaying lethality toward unrelated species. This can be utilized to the advantage of commercial poultry production, provided further research is conducted on characterizing whether this toxicity also occurs toward beneficial bacteria.

Conclusions: Impact on Poultry Industry and Future Directions

In the search for a replacement to antibiotic growth promoters, the poultry broiler industry has two main objectives, a substance that (1) increases the growth of broiler chickens (body weight gain and feed conversion ratio) and (2) prevents the colonization of invading pathogens. Ideally, a single feed additive would prevent pathogen colonization while developing beneficial microbiota to aid in bird growth and feed conversion (262). Multiple feed additives

have been attempted: antimicrobial agents, probiotics, prebiotics, and prebiotic-like substances. Probiotics need to be clearly identified and carefully analyzed to understand the influence they may have on the poultry GIT microbiota. As discussed previously, lactobacilli and bifidobacteria are two known groups that provide the host health and well-being based on their end products. These bacteria both ward off pathogens by creating an unfavorable environment against pathogen retention in the gut and also generally aid host GIT health, in turn resulting in enhanced bird growth (133).

To increase the efficacy of supplying probiotics to the host, the concept of synbiotics has been suggested. Synbiotics entail equipping the beneficial bacteria with substrates specific to their metabolic needs (23). Potentially, this allows for the greatest impact as it reduces the substrates taken by the probiotics from the host. Prebiotic-like substances are often times non-selective, therefore, combining a probiotic and a prebiotic-like substance does not fit into the synbiotic definition (263). Understanding the effects and specificity of probiotics, prebiotics, and prebiotic-like substances will allow for the best match of known commensal bacterial communities and substrates for a given host.

Yeast cells and YC products developed thus far have been extensively examined for their effects as supplements in animal feed. Numerous studies report the positive association with growth performance, immunostimulation, and microbiome modulation in animals and humans (209). In addition to being explored for their positive impacts as supplements in animal feed, yeasts and their derivatives have been investigated for their low risk and assurances of safety in their usage. Yeasts are cost efficient in both production and formulation (200). They do not have the ability to transfer genes they may acquire to pathogenic or commensal bacteria, or to the host. Yeasts are able to resist acquisition of antimicrobial resistance as well as not allowing for the

transfer of such resistance (209). This also allows yeast to be safely used in parallel with antibacterial agents. Yeasts also have multiple mechanisms of action, allowing them to be productive in a range of environments (200).

A more thorough understanding of the microbiome can elucidate the mechanisms of prebiotics and prebiotic-like substances. The GIT microbiome is distinct and unique in its functionality relying on the presence of a definable, and potentially identifiable, microbial consortia. Understanding the influences of the members of the microbiome and also the microbiome as a single entity will allow for a more directed approach in the search of therapeutics and growth promoters. The GIT microbiome may be more appropriately considered as an additional organ; it has impact on host growth and development, and host health.

The limitations in previous research conducted have made future research necessary to resolve unanswered questions. It is imperative to define universal and standardized detection methodology to identify the bacterial communities present in the healthy, mature poultry microbiome. This would alleviate the issue of having varying results based on detection methods utilized. In addition, evaluating the currently suggested probiotic candidate organisms (Table 1.3) indicates the potential advantages of involving multiple potential probiotic bacterial and yeast strains to exhibit a concerted effort in maintaining GIT health. This would allow for the identification of potentially more uniform mixed probiotic cultures consisting of functionally well-defined individual bacterial members that when used to inoculate newly hatched chicks ensures more rapid development of a mature beneficial microbiome.

Further work with yeast, YC, and yeast extracts needs to be conducted on poultry. Much of the discussion in the current review was based on the results from yeast products applied to animals and humans but not poultry. To gain an accurate sense of the effects in poultry, such

experimentation needs to be conducted in poultry (*in vitro* and *in vivo*). Additionally, many of the metabolites mentioned previously were investigated independent of yeast, YC, or yeast extract. It would be beneficial to assess the impact of metabolites and components from yeast individually as well as when combined. This would allow for the identification of beneficial metabolites and their respective individual and combined functional impacts on the corresponding host.

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Table 1.1: Commonly researched feed additives for host health, including growth promotion and pathogen prevention, used in animal feed, their modes of action, and reviews for reference.

Compound	What they do	How they work	Reviews for reference
Prebiotic	Food ingredient to act as substrate for beneficial bacteria in the host GIT microbiota	Host consumes prebiotic and it endures through the GIT relatively intact to the lower intestines where it selectively acts as substrate for beneficial bacteria	22- 24
Probiotic	Live microbial microbial feed supplements that beneficially impact intestinal microbial balance	Competes with pathogenic bacteria to colonize the intestines; ferments substrates to produce short- chain fatty acids; stimulated the immune response of the host.	23, 25, 26
Mannan-oligosaccharide	Specific oligosaccharide that inhibits pathogenic bacteria from binding the mucosal epithelial lining	Pathogens have receptors specific for mannan residues, the pathogenic bacteria binds the manna and does not bind to the host epithelial cells.	27- 29
Organic acid	Reduce the number of pathogens	Undissociated form traverses the bacterial cell membrane; once inside the bacterial cell, the organic acid dissociates to produce H+ ions, which lowers the pH. The bacterial cell then has to expend its energy to restore its natural balance rather than promote its own growth.	30- 34

Table 1 .2: Research conducted on commensal bacteria in the poultry GIT based on location.

Host	Site(s)	Age(s)	Commensal or pathogenic	Method of investigation	Reference
Chicken	Ileum, cecum	7, 13 days	Commensal	PCR based DGGE; 16S rRNA gene library analysis; qPCR	69
Chicken	Cecum	4, 8, 14, 21, 35 days	Commensal	DGGE; RFLP	6
Chicken	Ileum, cecum	4 wk	Commensal	Percent G+C profiling	70
Chicken	Cecum, intestines	4, 14, 25 days	Pathogenic	Primer (species-specific) of 16S rDNA	71
Chicken	Cecum	1 day; 1, 2, 4, 6 wk	Commensal	TGGE; 16S rRNA gene sequencing	64
Chicken	Crop, ileum, cecum, rectum	40, 41 days*	Commensal	16S rDNA sequencing	72
Chicken	Ileum, cecum	28 days	Commensal	FISH with 16S rRNA oligonucleotides	73
Chicken	Crop, duodenum, colon	2 mos	Commensal	FCM-FISH	74

* Indicates differing rearing methods: conventionally raised and organically raised, respectively.

Table 1 .3: Suggested microorganisms for potential probiotic use based on various characteristics.

[a]Mixed culture composed of 29 cecal bacterial strains that have shown to inhibit *Salmonella* colonization.

Microorganism	Host	Site isolated	Rationale	Reference
Enterococcus faecium	Chicken	Intestines	Bacteriocin-producing ability	135
Pediococcus pentosaceus	Chicken	Intestines	Bacteriocin-producing ability	135
Mixed culture[a]	Chicken	Cecum	Inhibition ability of *Salmonella*	99, 136- 139
Lactobacillus reuteri	Chicken	GIT	β-glucanase gene enhances growth and nutrient digestion	140
Lactobacillus fermentum	Chicken	GIT	Intestinal adherence, pathogen inhibition, tolerance to gastric enzymes	141
Bifidobacterium longum	Chicken	GIT	Anti-Campylobacter activity	142
Streptococcus faecium	Chicken	GIT	Impacts of body weight, feed conversion, carcass yield, *Salmonella* colonization	143
Streptococcus bovis	Cattle	Rumen	Inhibition ability of *Salmonella*	144

Table 1 .4: Published reviews on the considerations of common prebiotics in various hosts.

Prebiotic	Considerations	Host	Reference
Inulin-type	Structure overview	Not applicable	172
Short-chain carbohydrates	Gut function and health	Human	175
Inulin-type	Bifidogenic, resistant to digestion	Non-specific	176
Resistant starch	Production of SCFA, microbiome modulation, gut-associated immunomodulation	Human	177
Mannan-oligosaccharide	Modulation of gut microbiome	Poultry	27
Fructo-oligosaccharide, galacto-oligosaccharide, lactulose	Criteria for prebiotic classification	Human	168
Inulin-type, oligofructose	Quantification of inulin and oligofructose in Western diet	Human	173
Fructo-oligoaccharide	Bifidogenic, lack of carcinogenic and toxic effects	Poultry, Swine	174
Fructo-oligosaccharide, inulin-type	Selective to beneficial bacteria, prevent pathogen colonization	Poultry	178

2. Original-XPC™ effect on *Salmonella* enterica serovar Typhimurium and cecal microbiota from three different ages of birds when incubated in an anaerobic *in vitro* culture system

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ABSTRACT

Biological supplements are utilized in the poultry industry as a way to improve growth performance and reduce pathogen inhibition. Some of these supplements, for example prebiotics, are directed at working in concert with and in support of the microbiome of the lower intestinal tract of poultry. The current research evaluates the effects of Original-XPC^{TM} (XPC), a product with activity hypothesized to be similar to prebiotics, on *Salmonella* survival in the cecal microbiome as well as modulation of the cecal microbiota via an anaerobic *in vitro* mixed culture assay. Cecal slurries from three individual birds at each of three sampling ages (14, 28, and 42 d) were generated and allowed a 24 h pre-incubation period with the various treatments: XPC (1% XPC with basal poultry diet + cecal slurry), negative control (NC with basal poultry diet + cecal slurry), XPC + Feed (1% XPC with basal poultry diet), and cecal only (cecal slurry). The XPC, NC, and XPC + Feed were all challenged with *Salmonella* enterica serovar Typhimurium and subsequently plated on selective media at 0, 24, and 48 h. Analysis of the cecal microbiota indicated increased species diversity and richness directly related to sampling age, while no significant differences were observed among treatments. However results revealed treatment with XPC significantly reduced the survival of *S.* Typhimurium at the 24 h plating timepoint for both the 28 and 42 d sampling ages; while *S.* Typhimurium reduction in the NC appeared to eventually reach the same level by the 48 h plating timepoint. These findings suggest that 1) XPC may be capable of accelerating the rate (24 h versus 48 h) at which the cecal microbiota is able to limit *S.* Typhimurium growth and 2) the maturity of the cecal microbiota may be critical in limiting *S.* Typhimurium in the ceca. The results observed may attest to the early age of the bird at which XPC needs to be supplemented in order to maximize efficiency.

INTRODUCTION

Prebiotics are often used in the poultry industry as a replacement of antibiotic growth promoters; they are expected to maximize growth, while minimizing pathogen invasion by selectively stimulating only beneficial bacteria (Roberfroid, 2007). Prebiotics are defined as substances that travel past the upper gastrointestinal tract (GIT; resisting hydrolysis by gastric enzymes and degradation by acidic pH) remaining intact and acting as selective substrates for beneficial bacteria in the lower GIT in order to improve host health (Gibson and Roberfroid, 1995; Roberfroid, 2007). However, the definition of a prebiotic is continually evolving as more becomes understood about the gastrointestinal microbiome (Hutkins et al., 2015). With this in mind, there are several food ingredients available that do not fit the stringent prebiotic definition as set by Gibson and Roberfroid (2005), yet provide advantageous influences to the host health. These food ingredients are known as prebiotic-like compounds (Roto et al., 2015). A common prebiotic-like compound is a *Saccharomyces cerevisiae* fermentation product (SCFP), which contains the fermentation products of *S. cerevisiae* along with metabolites, the media used in the fermentation, yeast cell wall fragments (including mannon-oligosaccharides and β- glucans), and residual live yeast cells (Shen et al., 2011). Original-XPC™ (XPC; Diamond V Mills, Cedar Rapids, IA) is a commercially available product similar to SCFP however it lacks any residual live yeast cells, allowing for an extended shelf life (personal communication, Diamond V Mills). Research regarding XPC has been conducted in several different animal model systems, both *in vivo* and *in vitro*, to investigate its effects on host health (Gao et al., 2008; Osweiler et al., 2010; Price et al., 2010).

The compound XPC potentially falls into the category of prebiotic-like compounds. The mechanism of action is unclear, although it is suggested to have some immunological effects that

enable the host to ward off pathogen invasion. The current research utilizes an anaerobic *in vitro* mixed culture assay to mimic the chicken hindgut in order to quantify *Salmonella* survival. The *in vitro* methodology allows for a more direct assessment of the performance of XPC, while reducing confounding host variables (for example, host immune response) and being more cost efficient (Polli, 2008). The study utilized three different sampling ages in order to observe the temporal effect on the cecal microbiota that has been reported in previous studies (Scupham, 2009; Danzeisen et al., 2013; Oakley et al., 2014). Additionally, samples were taken at various time points for cecal microbiota analysis in response to treatment with XPC. In this study, we examine the potential of XPC treatment in the inhibition of *Salmonella* in the poultry host ceca and attempt to identify the mechanism of XPC. Furthermore, the current research characterizes the cecal microbial populations and evaluates the species diversity within the cecal microbiota as a result of XPC treatment and host maturity.

MATERIALS AND METHODS

Experimental Design

This experiment contained two trials, each with three biological replicates (individual birds) utilized at each of the three time points: 14, 28, and 42 days.

Salmonella Typhimurium Preparation

This study used a chicken isolate of *S.* Typhimurium (strain ST 97) resistant to nalidixic acid (NA) to selectively identify this specific strain from a mixed microbial background. Bacteria were grown in 6 mL Luria Bertani (LB) broth with 20 μg/mL NA for 16 h with shaking at 37°C. Bacteria was washed in phosphate-buffered saline (PBS) three times and resuspended in 1 mL PBS. Optical density was measured at 600 nm with a spectrophotometer (Beckman Coulter Inc, Brea, CA).

Broiler Chicken and Cecal Preparation

Animal handling and procedures conducted were in accordance with the guidelines of the University of Arkansas's Institutional Animal Care and Use Committee (IACUC). Ten male broiler chicks (per trial) were obtained from Cobb-Vantress, Inc. (Siloam Springs, AR), grown in a pre-disinfected Horsfall unit, and provided antibiotic-free corn-based poultry feed and water *ad libitum* until 14, 28, and 42 days of age. Broilers were randomly tagged with leg bands, euthanized by CO_2 asphyxiation, and their ceca were collected aseptically into sterile sample bags (VWR, Radnor, PA). The ceca were transferred into an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI), the cecal contents (0.1 g) were weighed, and subsequently diluted to 1:3000 in anaerobic dilution solution (ADS; 0.45 g/L KH_2PO_4, 0.45 g/L $(NH_4)_2SO_4$, 0.9 g/L NaCl, 0.1875 g/L $MgSO_4$-$7H_2O$, 0.12 g/L $CaCl_2$-$2H_2O$, 1mL/L 0.1% resazurin, 0.05% cysteine-HCl, and 0.4% CO_2-saturated sodium carbonate). The ADS was prepared as originally described by Bryant and Robinson (1961), with cysteine-HCl added prior to autoclaving as described in Shermer et al. (1998).

***In Vitro* Incubation**

The *in vitro* procedure was carried out as described previously (Donalson et al., 2007). Autoclaved serum bottles (100 mL) were prepared containing 0.5 g Torres Chick Starter (Table 2.1) and 1% XPC. A 40 mL volume of diluted cecal contents was added to each serum bottle. All serum bottles were placed directly into incubation at 37°C for a 24 h pre-incubation and inoculated with *S.* Typhimurium at a final concentration of 10^7 bacteria/mL after the pre-incubation. Contents were subsequently plated on LB + NA + novobiocin (NO) media to serve as the baseline (0 h incubation). Repeated plating occurred at 24 h and 48 h to determine *S.* Typhimurium survival. Each treatment group containing cecal contents, feed, and XPC (XPC)

was compared to three control treatments: 1) negative control (NC), 2) cecal only control (CO), and 3) XPC + Feed control (Table 2.1). The XPC + Feed control was added as a treatment after the 14 d sampling age in trial 1 to establish whether XPC was working alone or in concert with the cecal contents in the inhibition of *S.* Typhimurium. Aliquots of samples (2 mL) were collected at 0, 6, 12, 24, and 48 h for microbiome analysis for NC, CO, and XPC treatments.

Microbiome Analysis via Illumina MiSeq

Extraction of cecal DNA from aliquots of samples for microbiome analysis was conducted via QIAamp Fast DNA Stool Mini Kit according to the manufacturer's protocol (Qiagen). The final step of the DNA extraction deviated from the manufacturer's protocol which used DNase/RNase-Free distilled water in place of the elution buffer provided. Concentrations and purity of the cecal DNA samples were measured using the Nanodrop ND-1000 (Thermo Scientific, Waltham, MA).

Polymerase chain reaction (PCR) was used to amplify the V4 region of the 16S rRNA gene with dual-indexed primers with an Eppendorf Mastercycler pro S (Eppendorf, Hamburg, Germany) according to the methodology described in Kozich et al. (2013). Confirmation of the amplification and size of PCR amplicons was conducted on 1% agarose gel. Invitrogen SequalPrep kit (Life Technologies, Carlsbad, CA) was utilized for the normalization of PCR amplicons according to the manufacturer's protocol. The samples (5 µL of each sample) from each well were pooled together. For quantification of the pooled samples, the Eppendorf realplex Mastercycler ep gradient S (Eppendorf, Hamburg, Germany) was utilized with the KAPA Library Quantification Kit (KAPA Biosystems, Wilmington, MA) according to the manufacturer's protocol (from Ct values of standard curve $R^2 = 0.999$; efficiency = 96%). The length of the amplicon fragments was evaluated using the Agilent Bioanalyzer. Amplicons were

diluted to 4 nM with 0.2 N fresh NaOH and HT1 buffer, combined with prepared PhiX Control v3 (5%), and a final concentration of both reagent and library was produced at 6 pM. The index primer, Read 1 and Read 2 sequencing primers, and the sequencing library were subsequently loaded into an Illumina MiSeq reagent cartridge.

Sequence and Statistical Analysis

Sequencing (FastQ) files were downloaded from the Illumina Basespace website consisting of both demultiplexed R1 and R2 sequencing reads (each one being approximately 250 bp in length). Samples with less than 10,000 reads were excluded from analysis. Sequence analysis using the Greengenes database as the reference database, classification of operational taxonomic units (OTUs) among sequences sharing 97% identity, and species diversity and richness (Chao1 and Shannon Diversity Index) were evaluated via the Quantitative Insights Into Microbial Ecology (QIIME; Caporaso et al., 2010) pipeline. UniFrac principal coordinates analysis (PCoA) plots, generated via QIIME, were used to determine the multidimensional distances reflecting similarities and differences between samples based on age and treatment.

The JMP® Pro 12 (SAS Institute, Cary, NC) software was utilized for statistical analysis. One-way analysis of variance (ANOVA) and student's t tests evaluated the statistical significance among microbial abundance data with a level of significance of less than 0.05.

RESULTS AND DISCUSSION

S. Typhimurium Survival in Treatment with XPC

The first objective of the current research used the fermentation product, XPC, to examine its impact on *S.* Typhimurium in a mixed culture assay. The poultry ceca contain the largest number of bacteria due to the relatively slow digesta transit time (Salanitro et al., 1974). As the poultry host matures, the composition of the cecal bacteria become more diversified and

reach concentrations that allow them to maximize their metabolic fermentative activities in an anaerobic environment (Roto et al., 2015). The *in vitro* assay in the current study attempted to mimic the environment of the chicken ceca, providing chicken feed as the nutrient supply for the cecal contents while maintaining an anaerobic environment. The majority of the bacteria in the poultry ceca are strictly anaerobic, and have traditionally been enumerated using anaerobic jars and selective media (Fan et al., 1995; Ricke and Pillai, 1999).

When considering the definition of prebiotics as set by Roberfroid (2007), XPC should reach the lower GIT without being hydrolyzed or digested. The methods in the current study demonstrate the potentially synergistic effects between XPC and the cecal bacterial populations based on the assumption that XPC is maintaining its activity until it reaches the ceca. Based on previous studies, the assay utilized a 24 h adaptation period of each sample containing cecal contents and poultry feed (Donalson et al., 2007). This allows the cecal bacteria to ferment and continue metabolism of substrates from both poultry feed, and in some cases XPC treatment, prior to being challenged with *S.* Typhimurium (Donalson et al., 2007). The 24 h adaptation period is necessary for XPC to inhibit *Salmonella* due to its prebiotic-like mechanism (affects the microbial ecology of the gut).

Previous research has indicated the ability of XPC in animal cecal contents inhibit various pathogens as well as increase antibodies in blood samples (Jensen et al., 2008; Gao et al., 2008). The current study followed in suit, indicating 0.5 to 3.0 log reduction in *S.* Typhimurium survival in treatment with XPC compared to the NC treatment. At the 14 d sampling age, there were no significant differences in the *S.* Typhimurium survival observed in the XPC treatment when compared to the NC in either trial, although there were numerical (approximately 1.0 log) reductions (Figures 2.1A and B). The results obtained at the 28 d sampling age varied among

trials. All reductions observed in the XPC treatment were significant at the 24 and 48 h plating timepoints at the 28 d sampling age in both trials (Figures 2.1C and D). However, the reductions observed in the XPC treatment in trial 2 were approximately 2.0 and 3.5 logs greater at the 24 and 48 h plating timepoints, respectively, as compared to the trial 1. In the first trial at 28 d, the XPC treatments indicated approximately 1.0 and 3.0 log reductions in the *S.* Typhimurium recovered at both the 24 and 48 h timepoints as compared to the NC. While in the second trial at 28 d, the XPC treatment revealed *S.* Typhimurium population levels to be 2.0 and 3.0 logs lower than the NC treatment in the 24 and 48 h plating timepoint respectively, with the recovery below the limit of detection of 10 CFU/ml (LOD) at the 48 h plating timepoint (Figures 2.1C and D). The variation observed between the two trials at the 28 d sampling age is potentially an indicator to the degree of microbial development (and, in turn, inconsistency) in the cecal microbiome. This may suggest that treatment with XPC, similar to other products intended to support the cecal microbiome and the immune system, may need to be administered at an earlier age in order to be effective in its reduction in *S.* Typhimurium (Sharma and Burmester, 1982; Nisbet, 2002; Bielke et al., 2003).

At the 24 h plating timepoint for the 42 d sampling age, there were numerical reductions (between 1.0 and 2.0 logs) in both trials when comparing the XPC treatment and NC, with trial 2 trial indicating significant differences (Figures 2.1E and F). By the 48 h plating timepoint at the 42 d sampling age, there were no significant differences observed among any treatments, with the XPC treatment being below the LOD in both trials. What is interesting is that although there were numerical differences between the XPC and NC treatments, both treatments contained cecal contents and were able to reduce the level of *S.* Typhimurium present to the LOD by the 48 h plating timepoint. This suggests the potential of the cecal microbiome adapting to the

environment and being able to possibly out compete *S.* Typhimurium for nutrients and/or generate an unfavorable environment by the production of short chain fatty acids (SCFA; Fooks and Gibson, 2002).

The XPC + Feed control (containing no cecal slurry) indicated higher *S.* Typhimurium recovered compared to all treatments (XPC, NC, and CO) at all timepoints (14, 28, and 42 d; data not shown). The comparison of the results of the XPC treatment to the control containing only XPC + Feed control suggests the necessity of the cecal contents to exercise the mechanistic activity of XPC. The XPC + Feed control (containing no cecal slurry) revealed a 3.0 log reduction in the abundance of *S.* Typhimurium from the 0 h plating timepoint to the 48 h plating timepoints across all ages, while the XPC treatments (containing cecal slurry, feed, and XPC) in both trials exhibited much greater total log reductions (4.0 to 6.0 logs; Figures 2.2A to E).

Cecal Microbiome Analysis

In poultry, the most vulnerable time in the maturation to market age is early on in life when the intestinal tract is continuing to change both anatomically and physiologically, as well as the establishment of various bacterial strains along the intestinal epithelium (Schleifer, 1985; Iji et al., 2001; Brisbin et al., 2008). The stability of the cecal microbiota is directly related to age, as suggested by the increased variability observed among cecal microbiota composition in younger chickens (14 days) when compared to more mature chickens (28 days; Torok et al., 2009). The transient bacterial populations in the cecal microbiota of younger animals indicate immaturity and potentially increased susceptibility to invasion by pathogenic bacteria while the stable diversity among cecal populations in a mature broiler GIT can lead to increased protection from pathogen invasion (Lozupone et al., 2012). Culture-independent analytical methods to characterize a given environment has become commonplace as it allows the study of the

microorganisms within that environment without prior culturing, thereby reducing the number of potential biases introduced by culture-based methods (Langendijk et al., 1995; Ricke and Pillai, 1999; Amit-Romach et al., 2004; Wooley and Ye, 2010). Sequencing of samples allows for the evaluation of the variation of both the species diversity as well as the structure of the communities over time and space (Hamady et al., 2010). The analysis of the 16S rRNA gene using primers targeting a hypervariable region (V4 in the current research) provides the unique properties of ubiquity while being conserved with a domain region of differing evolutionary rates, thereby making it an ideal phylogenetic marker (Case et al., 2007; Caporaso et al., 2011).

After filtering the sequences based on read quality and sample size, there were a total of 135 samples in each of the two trials (45 samples per treatment total: three biological replicates at three sampling ages, each with five microbiome sampling timepoints) of the V4 region of the 16S rRNA gene analyzed. Analytical information regarding the sequences generated revealed 25,013,102 and 20,856,668 total reads and error rates (as determined by the bases within the reads that align with three errors or less to the PhiX control sample included in the run) of 2.05 and 2.15% in the first and second trials respectively.

The most abundant phyla identified in both trials were in accordance with that of previous research (Salanitro et al., 1974; 1978; Wei et al., 2013): Firmicutes (trial 1: 68.06 %; trial 2: 52.27 %), Proteobacteria (trial 1: 28.06 %; trial 2: 15.30 %), and Bacteroidetes (trial 1: 1.04 %; trial 2: 30.13%; Figures 2.3A and B). Similar to the results in trial 2 of the current research, Firmicutes and Bacteroidetes have been identified as the most abundant phyla in the chicken cecal microbiota in culture-based studies (Salanitro et al., 1974; 1978; Figure 2.3B). The abundances of both Proteobacteria and Bacteroidetes in trial 2 as well as in previous studies are observed to be more variable (at the expense of the abundance of Firmicutes) with either

bacterial community ranging from less than 10% to greater than 30% abundance (Zhu et al., 2002; Wei et al., 2013). The largest variation among trials was observed in Bacteroidetes, in which the observed abundance in trial 1 was less than has previously been observed (Wei et al., 2013; Figure 2.3A). The variation observed between the trials suggest that there may have been uncontrolled environmental factors that resulted in distinct phyla abundances among all nine broilers utilized in trial 1 from the nine broilers used in trial 2. Trial 2 results are more typical of results observed within the lifespan of the cecal microbiota of healthy chickens (Zhu et al., 2002; Lu et al., 2003).

The order level analysis revealed Clostridiales to be most abundant in both trials relative to Enterobacteriales, Lactobacillales, and Streptophyta (Figures 2.4A and B). As could be expected, the Enterobacteriales were more abundant in trial 1 based on the large proportion of Proteobacteria at the phylum level, while in trial 2, the abundances of Lactobacillales and Enterobacteriales were more similar. At the family level characterization, *Enterobacteriaceae* (Proteobacteria phylum), *Lachnospiraceae* (Firmicutes phylum), and *Ruminococcaceae* (Firmicutes phylum) were the most abundant in both trials, with slightly increased *Enterobacteriaceae* abundance in trial 1 (Figures 2.5A and B). In a previous study, similar results, reported *Lachnospiraceae* to be most abundant, followed by *Ruminococcaceae* and *Enterobacteriaceae* in the cecal microbiota of chickens from 7 to 19 d (Videnska et al., 2013). Videnska et al. (2013) suggested that because infection with *S*. Enteritidis resulted in a decrease in *Ruminococcaceae* with a concurrent increase in *Enterobacteriaceae*, then *S*. Enteritidis could influence the composition of the cecal microbiota in chickens. The results in the current study are in agreement with this finding in the 14 d sampling age, which is within the range of sampling ages (7 to 19 d) taken by Videnska et al. (2013) (Figures 2.5A and B). It is possible that there

may be a degree of protection due to appropriate taxonomic balance based on the increased reduction of *S.* Typhimurium observed in trial 2 when compared to trial 1 (CO phylum and order abundances were not included in the figures as they were not challenged with *S.* Typhimurium and therefore unable to be correlated to reductions observed).

Analysis at the genus level indicated *Bacteroides*, an unknown genus belonging to the family *Enterobacteriaceae*, and an unknown genus belonging to the family *Lachnospiraceae* to be the most abundant in all samples analyzed in trial 2 (Figures 2.6B). Both sequences identified as being related to *Lachnospiraceae* and *Oscillobacter* were identified in the current study, which is in accordance with previous studies (Gong et al., 2008; Luo et al., 2013; Videnska et al., 2013; Oakley et al., 2014). The presence of both of these bacterial populations have been suggested to produce SCFA as well as play roles in the maintenance of the intestinal epithelial in different animal models (Lam et al., 2012; Lee et al., 2012; Oakley et al., 2014). Within each sampling age and treatment, differing genera were revealed to be most abundant. In trial 1, the unknown genus belonging to *Enterobacteriaceae* was most abundant through the sampling ages and treatments, while the *Bacteroides* (phylum Bacteroidetes) followed by *Oscillospira* (phylum Firmicutes) were most abundant in trial 2. The CO treatment exhibited the largest abundance of *Faecalibacterium* in both trials. As this treatment lacked a nutrient supply, these results suggest that *Faecalibacterium* may be capable of out-competing other bacterial communities in a nutrient starved environment. *Faecalibacterium* is recognized to have anti-inflammatory properties in human and murine models, and butyrate-producing properties in broiler supplemented with vitamins in their diets (Sokol et al., 2008; Louis and Flint, 2009; Luo et al., 2013).

The temporal effects on species diversity and richness as evaluated by Chao1 index, observed OTUs, and phylogenetic diversity (PD) revealed that all three measurements follow the

same trend of directly increasing with age in both trials (Figures 2.7A to C). Trial 1 revealed larger variation, with detectable decreased measures in the 14 d compared with the later sampling ages (28 and 42 d), while trial 2 revealed significant increases in the 42 d compared to both of the early sampling ages (14 and 28 d). Increasing cecal microbiota complexity directly related to sampling age has been observed in previous studies in poultry intestinal microbiome analysis (Salanitro et al., 1974; Danzeisen et al., 2013; Oakley et al., 2014). Meimandipour et al. (2011) provided evidence for this by the variation in the production of SCFA observed in the ceca across various sampling ages. The rarefaction curves for the number of observed OTUs and the Chao1 index indicated the 42 d sampling age to be significantly increased when compared to the 14 and 28 d sampling ages (Figures 2.7A and C). The continued projection upward rather than a plateau indicates that further subsampling of sequences would increase the species richness, increase the numbers of OTUs, and add new branches to the tree of sequences resulting in increased PD (Figures 2.7A to C; Figures 2.8A to C). The trend when evaluating the OTU and PD rarefaction curves revealed CO to consistently have higher measures, with a significantly increased value in the PD rarefaction curve for trial 1 (Figure 2.8B and C).

The Shannon diversity index in both trials revealed no significant differences within a given treatment among the sampling ages except in XPC treated samples. The overall Shannon diversity indices were higher for the NC and XPC treatments in trial 1 when compared to trial 2 (Table 2.2). What is interesting is that the XPC treatment exhibited the lowest Shannon diversity indices at the 14 d sampling age than any other treatment or sampling age in both trials, yet was the only treatment to significantly increase when compared to the 42 d sampling age. This may suggest the necessity of introducing XPC to the host at an earlier age in order to maximize the effects on cecal microbial diversity development. The increased diversity in the structure of the

cecal microbial communities is associated with weight gain at later stages of the market broiler lifespan (Lu et al., 2013). However, when considering that a broiler chicken does not begin puberty until 210 days of age, the broiler cecal microbiomes identified in recent studies are very young and therefore may not reach the optimum diversity for maximized broiler growth (Danzeisen et al., 2013; Lu et al., 2013; Oakley et al., 2014).

The UniFrac Principle Coordinate Analysis (PCoA) plots indicated significant differences in the weighted and unweighted plots when observing the influence of sampling age and treatment in both trials. There was no clustering visible in any of the weighted PCoA plots based on sampling age or treatment in either trial (Figures 2.9A to D). However in the unweighted plots there was distinct clustering visible in both the sampling age and treatment in both trials (Figures 2.10A to D). The unweighted PCoA plot revealed the CO control to cluster in both trials while there were two mixed groups consisting of mixtures of NC and XPC treatment suggesting there to be no distinguishable effect from treatment with XPC based on clustering (Figures 2.10A and C). Similar clustering was observed in the unweighted PCoA plots in trial 2 based on age, revealing 14 and 42 d samples to clustering into their own respective groups while 28 d samples were less clustered and overlapped into both 14 and 42 d clusters (Figure 2.10D).

CONCLUSIONS

Research conducted evaluating the effectiveness of feed additives seeking to fill the gap left as a consequence of the retraction of antibiotic growth promoters (AGPs) from animal feed are commonplace. What has been recognized in the current research along with previous work is that feed additives, aside from vitamins (Luo et al., 2013), are appearing to have little impact on the composition of the cecal microbiome in general (Denzeisen et al., 2013; Oakley et al., 2014). The current study reviewed the influence of XPC on the inhibition of *S.* Typhimurium and

reported that there was initial prevention, however the level of reduction eventually became equal among all of the treatments containing cecal contents. These findings suggest the ability of XPC to accelerate the rate at which *S.* Typhimurium and possibly other pathogens are inhibited by the cecal microbiota. Other studies more dramatically portray the influential abilities of XPC in various animal models, indicating an influential "host" factor (GIT morphology, immunologic response, growth performance, and pathogen reduction; Gao et al., 2008, 2009; Jensen et al., 2008; Osweiler et al., 2010).

There were significant observations regarding the successional changes in microbiome complexity that have not been previously observed with feed additives (Denzeisen et al., 2013; Lu et al., 2003; Oakley et al., 2014). With the shift in diversity, there may be related shift in the physiological functions performed by the microorganism present (Lu et al., 2003). Because antibiotic growth promoters have thus far indicated the ability to promote growth while limiting pathogens in poultry, it would be beneficial to characterize the intestinal microbiome when supplemented with AGPs, and compare the intestinal microbiome to that produced from various feed supplements (probiotics, prebiotics, synbiotics). For the poultry industry to utilize the current research, it may be beneficial to accelerate development of the intestinal microbial complexity of the broiler host by using supplements that interact with the host intestinal microbiome and promote a diversity related to its maturity.

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Table 2 .1: Ingredient composition of the Torres Chick starter diet.

Ingredient	Composition of Total (%)
Corn	63.07
Soybean meal	25.75
Fat	2.85
Calcium carbonate	1.03
Dicalcium phosphate	1.10
Salt	0.40
DL methionine 99.5	0.28
Trace minerals	0.10
Choline chloride 60%	0.22
Vitamin premix	0.20
ProPack	5.00

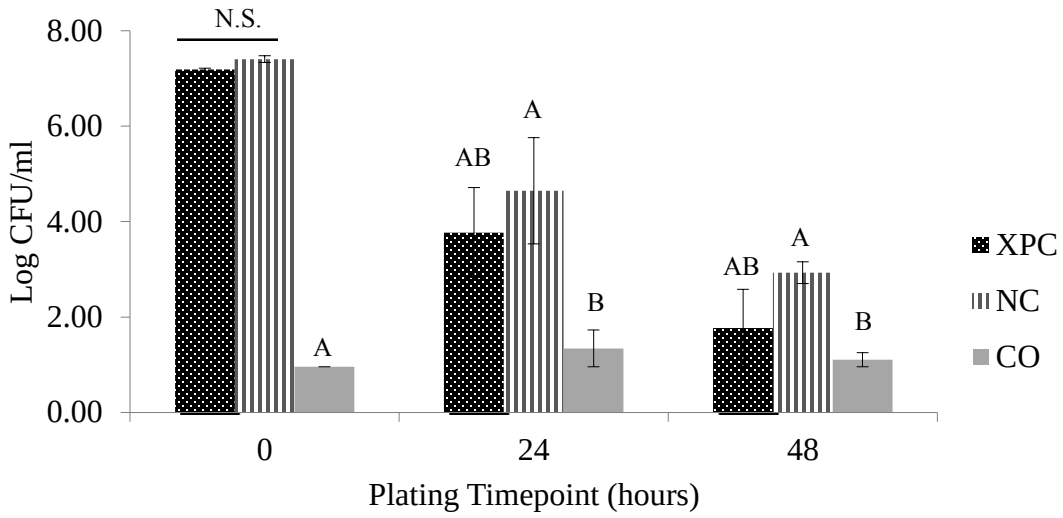
Table 2 .2: Shannon diversity index based on treatment and sampling age within their respective trials. Samples are analyzed for significant differences within a treatment at the various sampling ages.

		Sampling Age (days)		
	Treatment	14	28	42
Trial 1				
	XPC	1.89^{B}	2.31^{AB}	2.35^{A}
	NC	1.96	2.08	2.17
	CO	2.15	2.31	2.27
Trial 2				
	XPC	1.75^{B}	1.91^{AB}	2.04^{A}
	NC	1.92	1.82	1.92
	CO	2.17	2.21	2.28

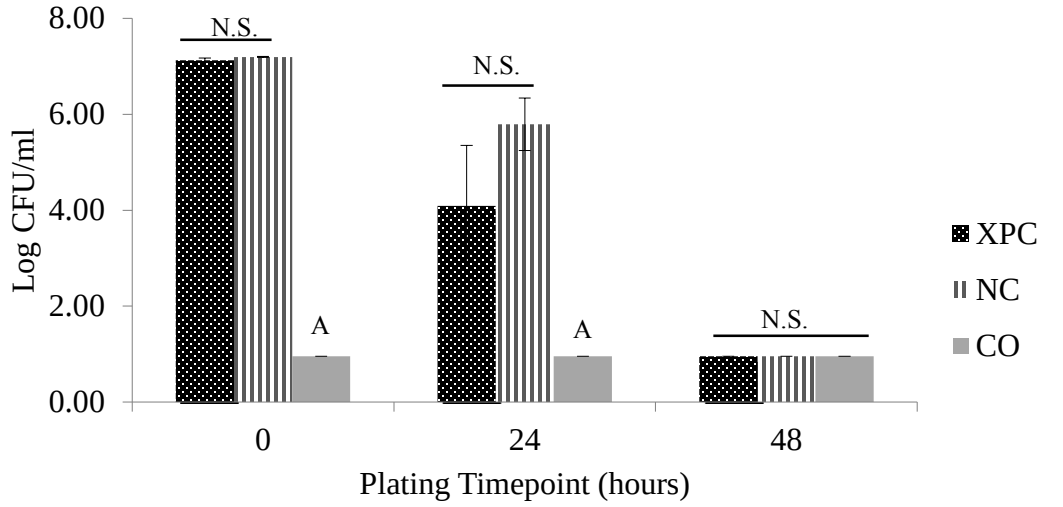
XPC = XPC treatment; NC = negative control; CO = cecal only control.
Differing letters within a treatment reveal significant differences ($P > 0.05$).
Lack of numbers indicates no significance differences among Shannon diversity indices within the treatment group.

Figure 2 .1A to F: S. Typhimurium survival amond treatments (XPC = XPC treatment; NC = negative control; CO = cecal only): A) trial 1- 14 day old chickens, B) trial 2- 14 day old chickens, C) trial 1- 28 old chickens, D) trial 2- 28 day old chickens, E) trial 1- 42 day old chickens, and F) trial 2- 42 day old chickens. Differing letters indicate significant differences ($P < 0.05$).

A)



B)



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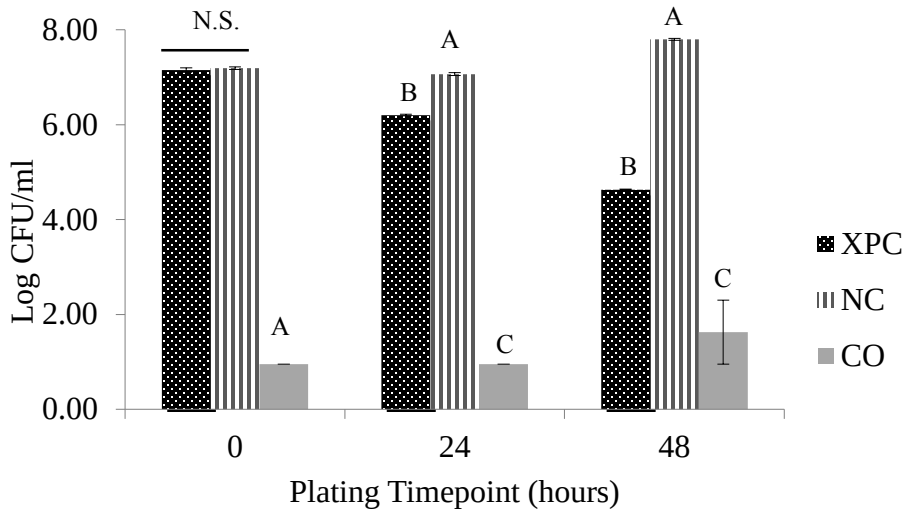
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Plating Timepoint (hours)
XPC
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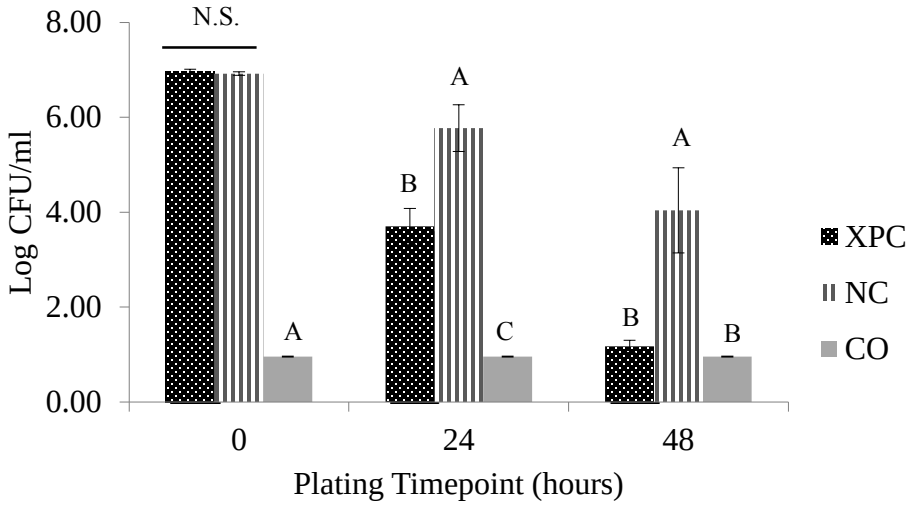
CO

D)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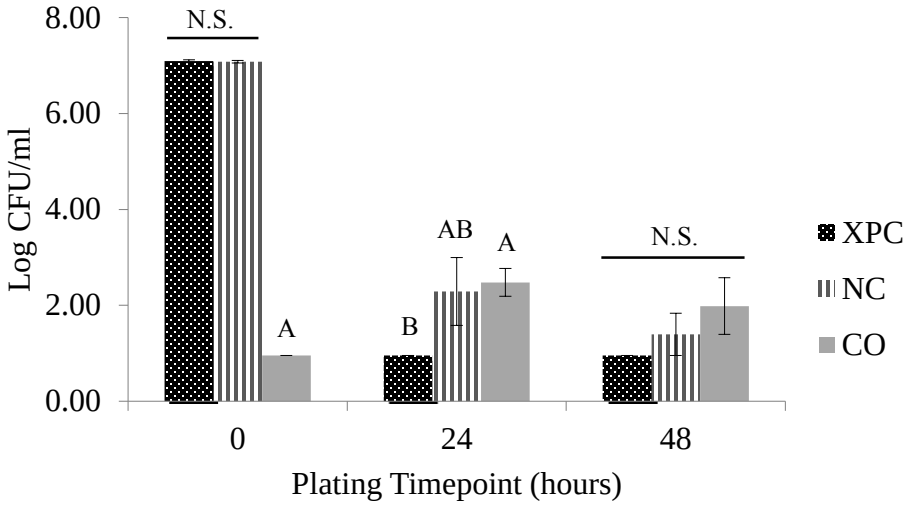
E)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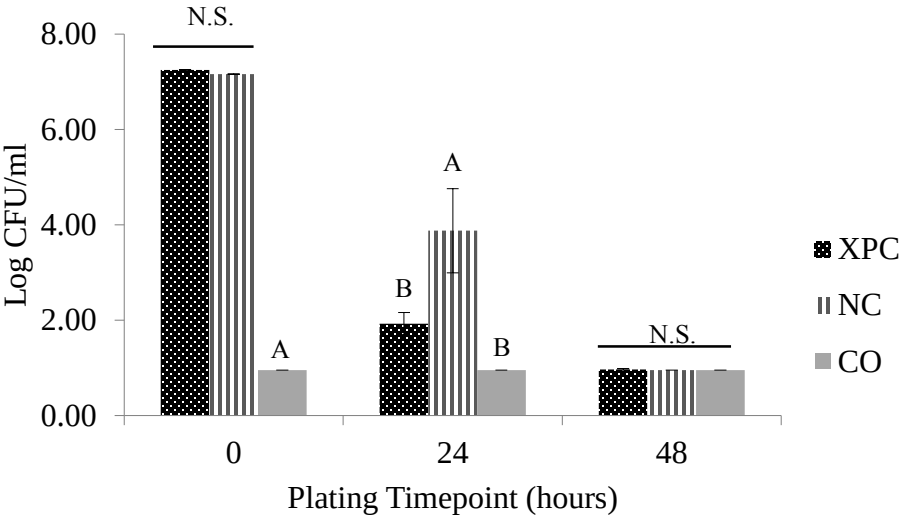
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Plating Timepoint (hours)
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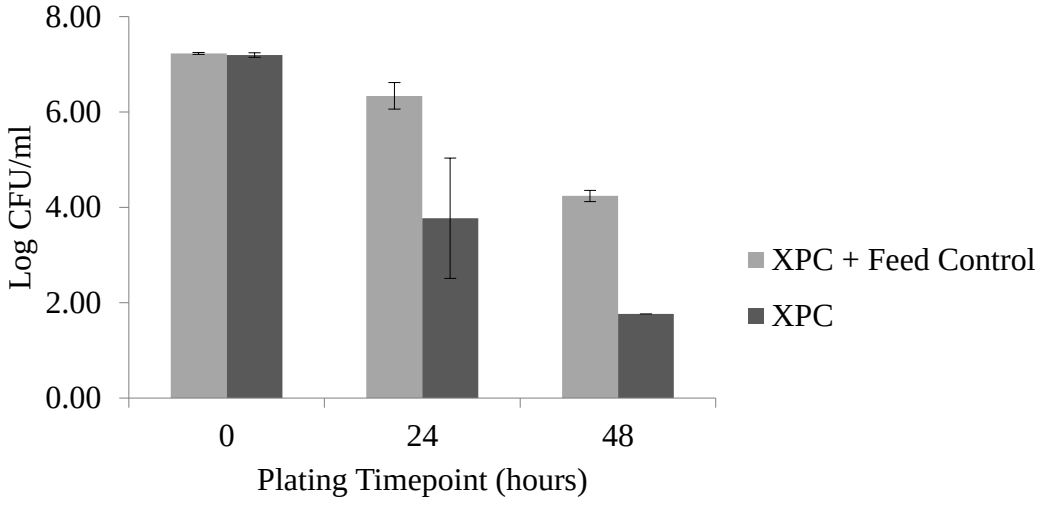
Figure 2. 1A to E: S. Typhimurium survival comparing XPC + Feed Control and XPC treatment: A) trial 2- 14 d chickens, B) trial 1- 28 d chickens, C) trial 2- 28 d chickens, D) trial 1- 42 d chickens, and E) trial 2- 42 d chickens. No figure for trial 1- 14 d chickens is presented as data for XPC + Feed Control was not collected at this timepoint.

A)



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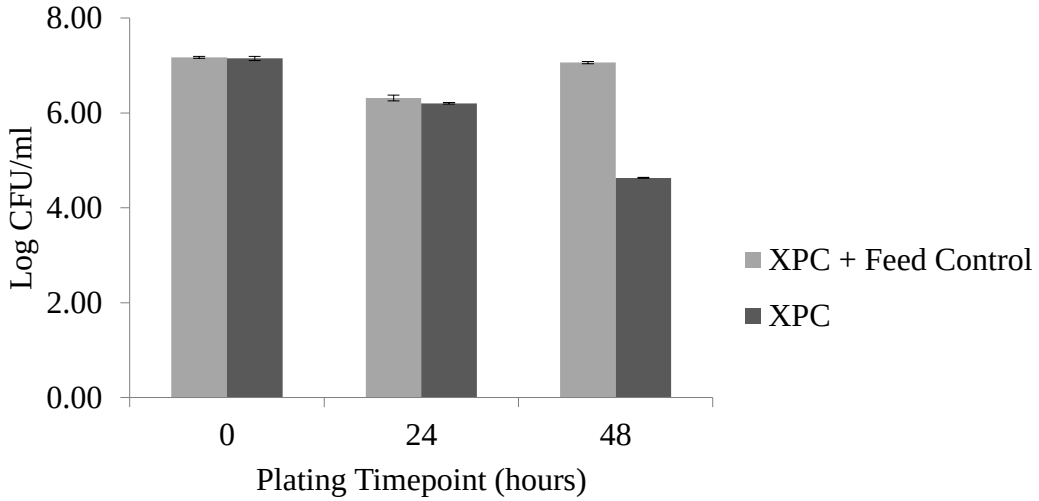

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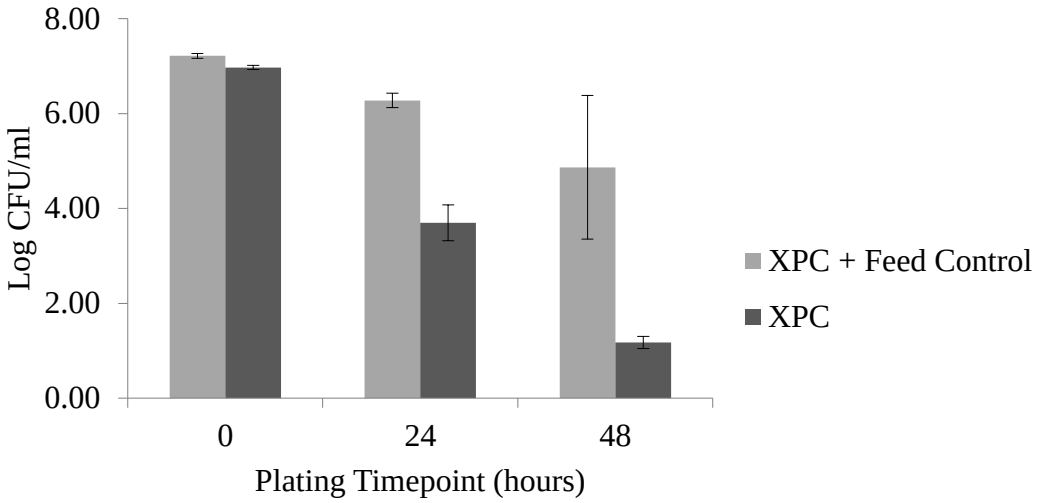
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Log CFU/ml
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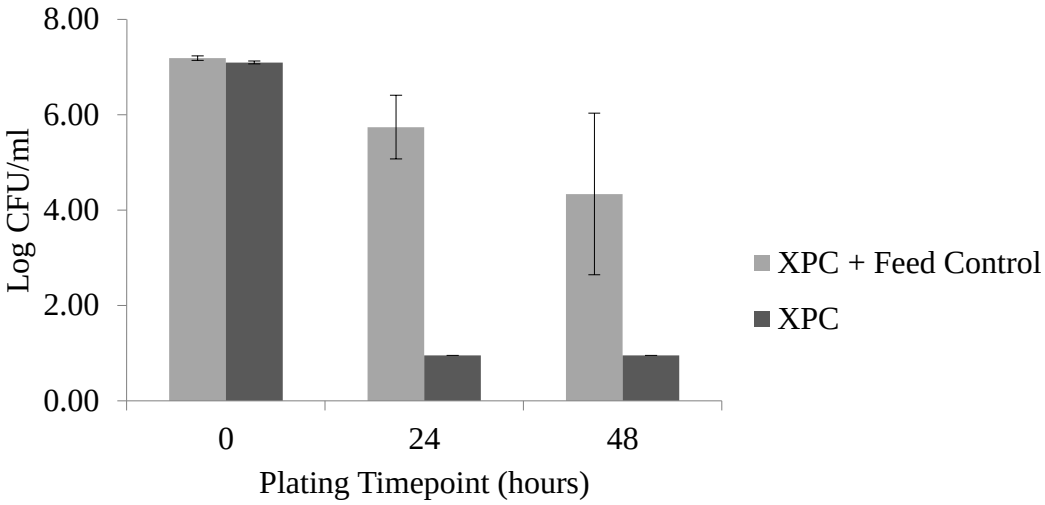
Plating Timepoint (hours)
XPC + Feed Control
XPC

D)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0.00
Log CFU/ml
0
24
48
Plating Timepoint (hours)
XPC + Feed Control
XPC

E)

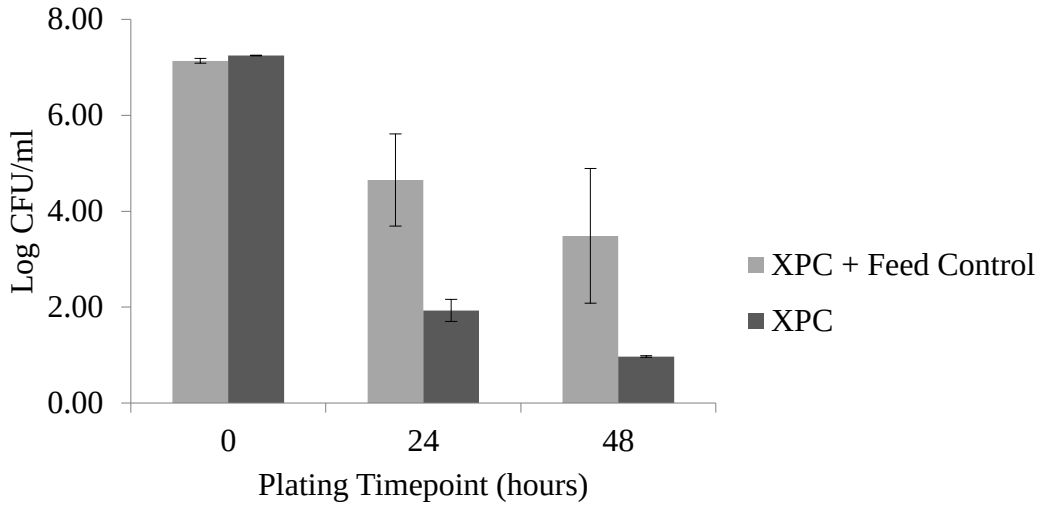

8.00
6.00
4.00
2.00
0.00
Log CFU/ml
0
24
48
Plating Timepoint (hours)
XPC + Feed Control
XPC

Figure 2. 2A and B: Relative abundances of Firmicutes, Bacteroidetes, and Proteobacteria based on treatments (NC and XPC) and sampling age in A) trial 1 and B) trial 2.

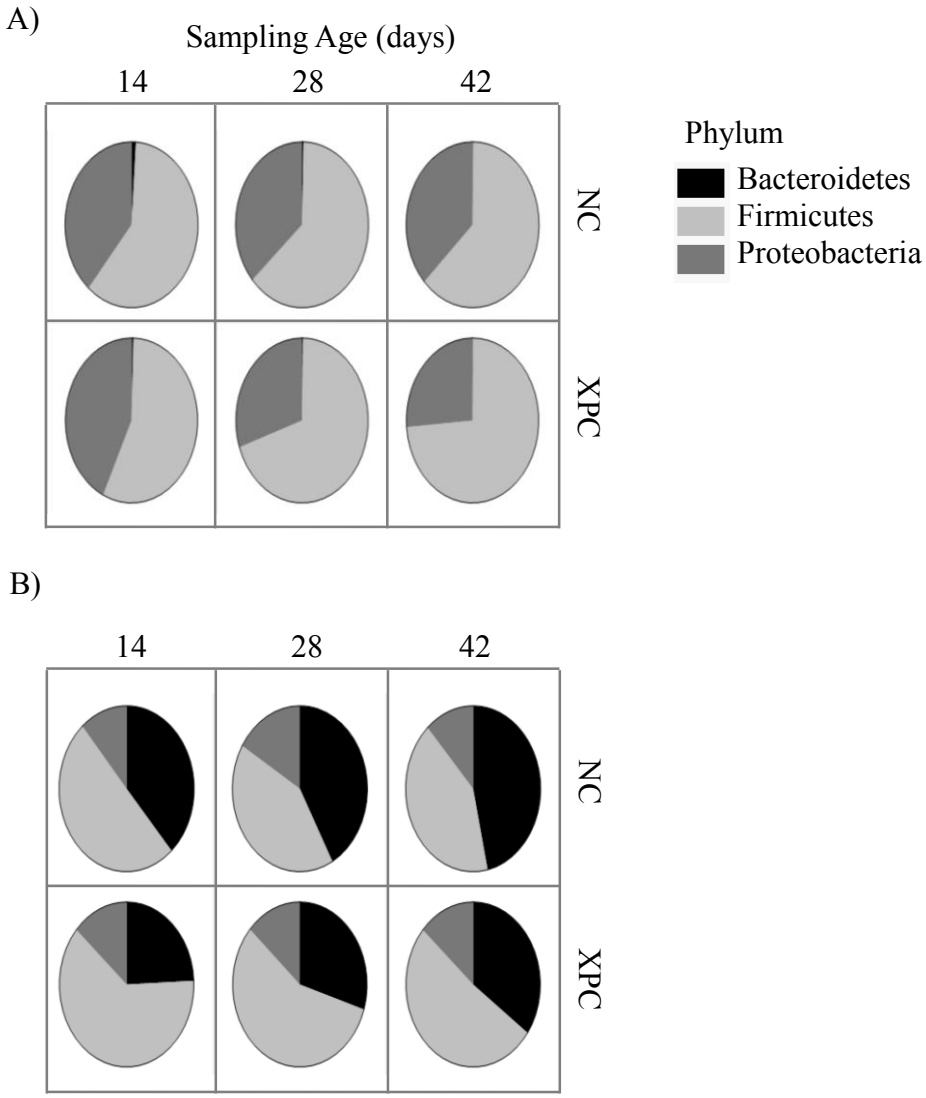


Figure 2 .3A and B: Relative abundances at the order level based on treatments (NC and XPC) and sampling age in A) trial 1 and B) trial 2.

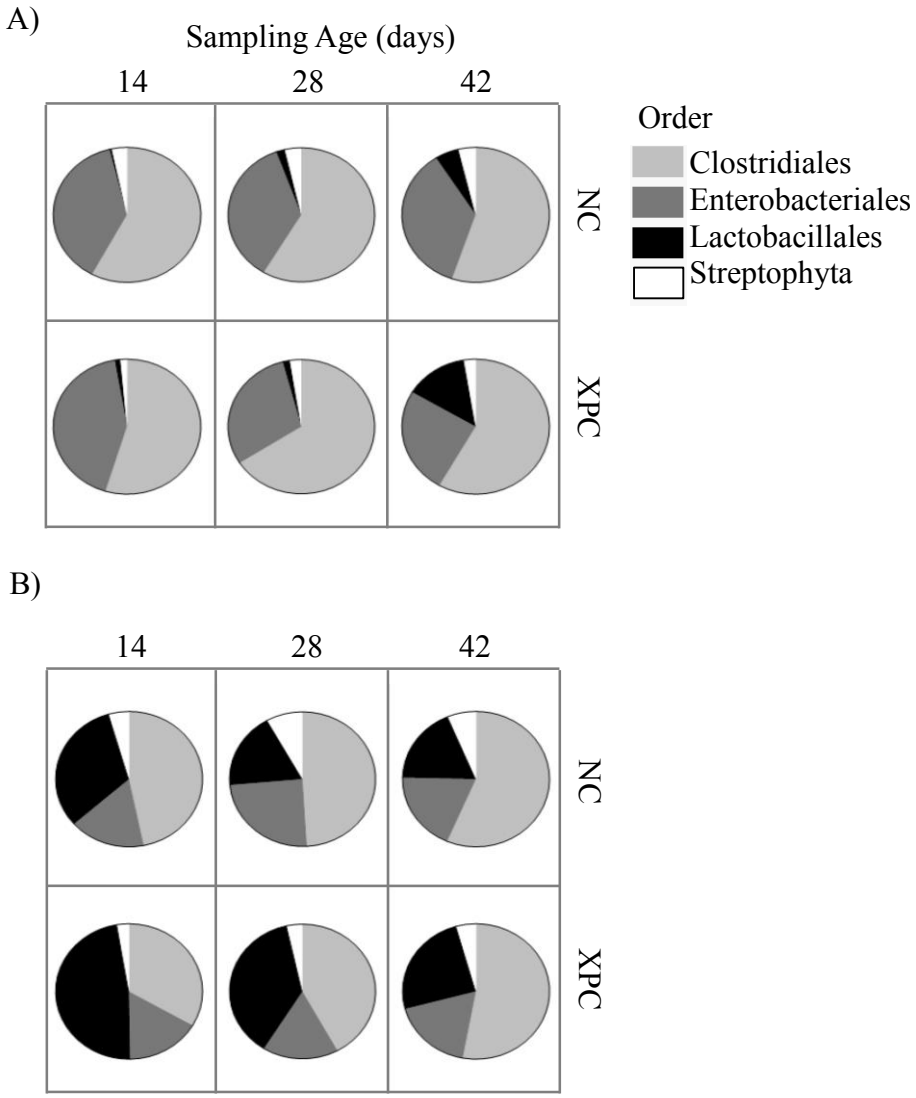


Figure 2 .4A and B: Relative abundances at the family level based on treatments (NC and XPC) and sampling age in A) trial 1 and B) trial 2.

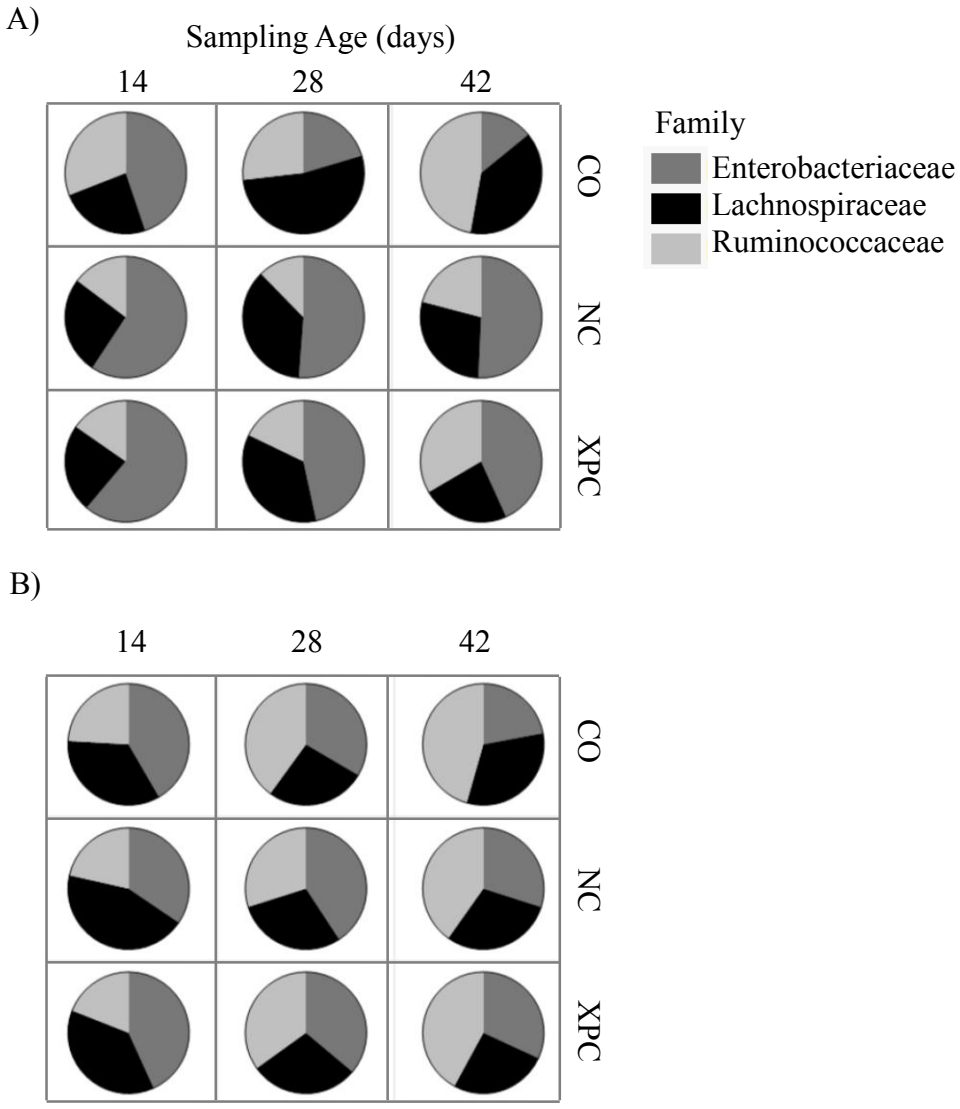


Figure 2 .5A and B: Genus level relative abundances of the cecal microbiota samples at various sampling ages among all treatments.

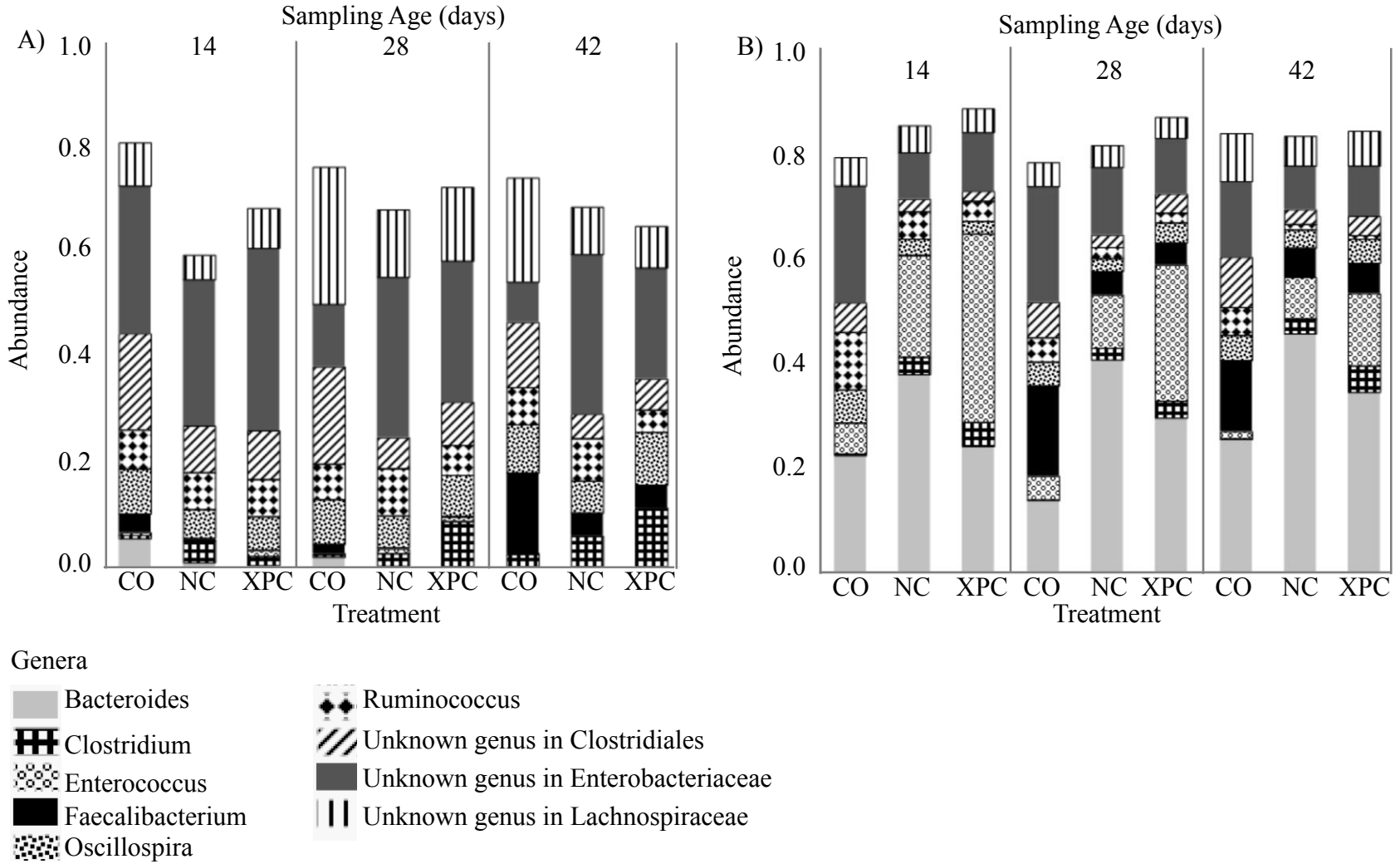


Figure 2 .6A to C: Rarefaction curves of cecal microbiome samples based on sampling age displaying A) Chao1 index measuring species richness, B) observed OTUs, and C) phylogenetic distance measuring phylogenetic diversity by sampling depth. Asterisk (*) indicates significant difference ($P < 0.05$).

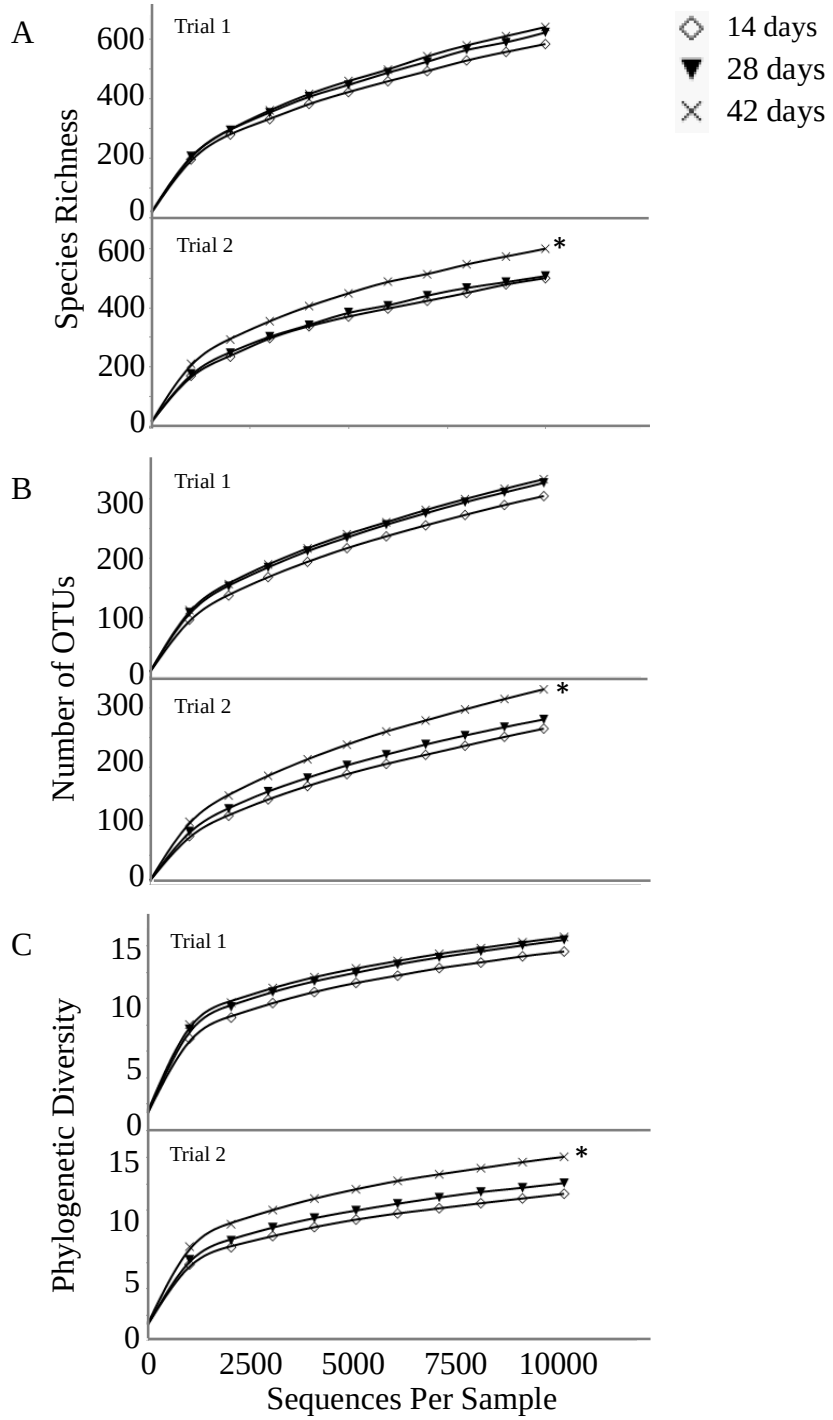


Figure 2 .7A to C: Rarefaction curves of cecal microbiome samples based on treatment displaying A) Chao1 index measuring species richness, B) observed OTUs, and C) phylogenetic distance measuring phylogenetic diversity by sampling depth. Asterisk (*) indicates significant difference ($P < 0.05$).

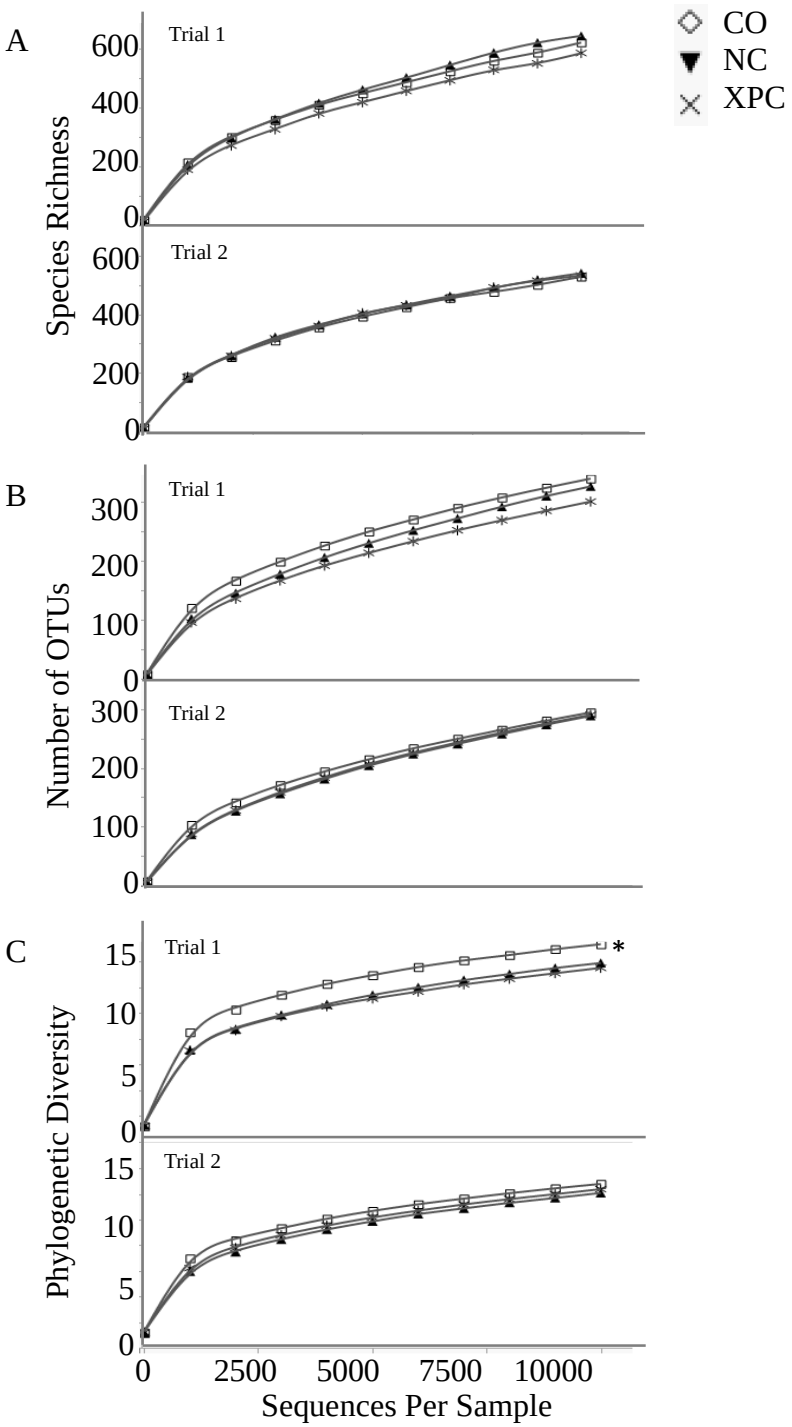


Figure 2 .8A to D: UniFrac weighted PCoA plots of A) trial 1 based on treatment, B) trial 1 based on sampling age, C) trial 2 based on treatment, and 4) trial 2 based on sampling age.

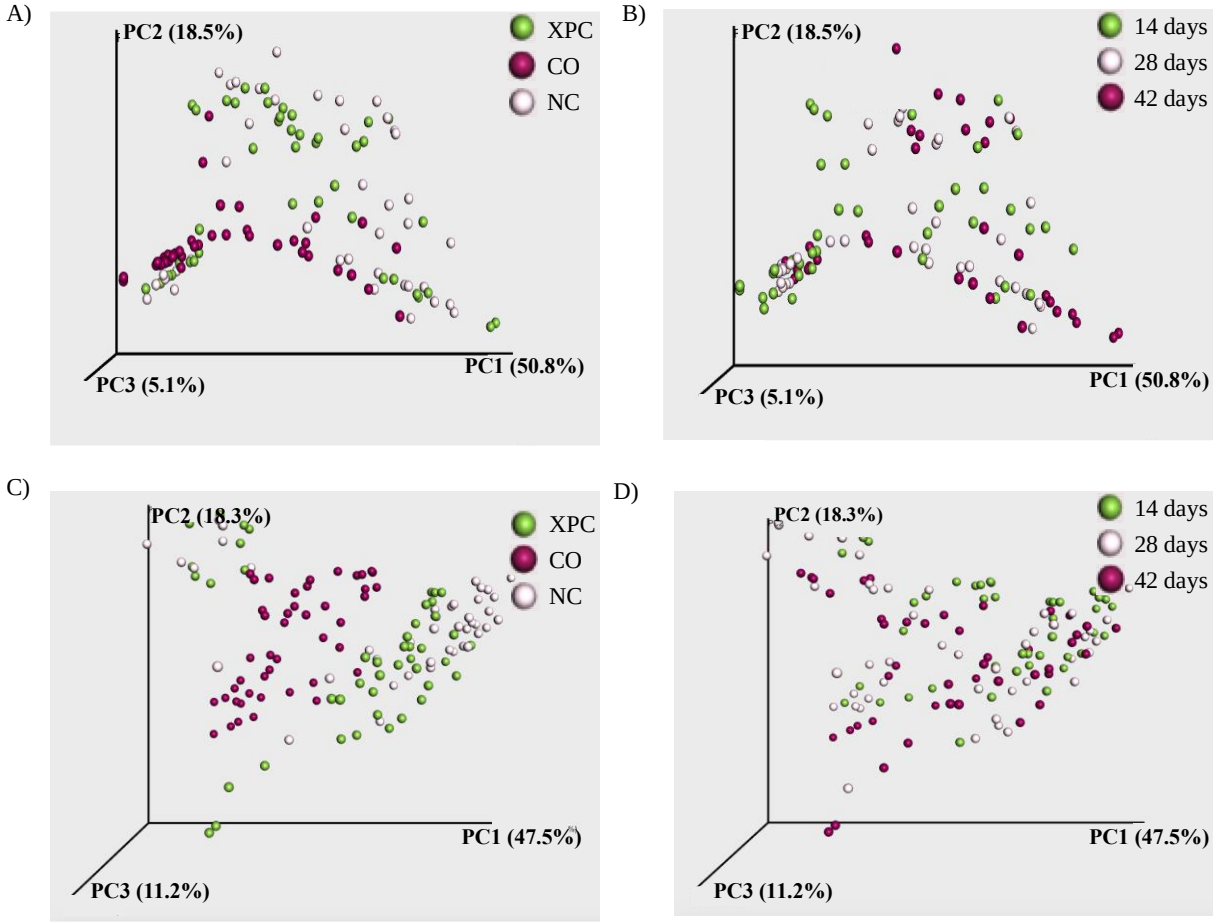
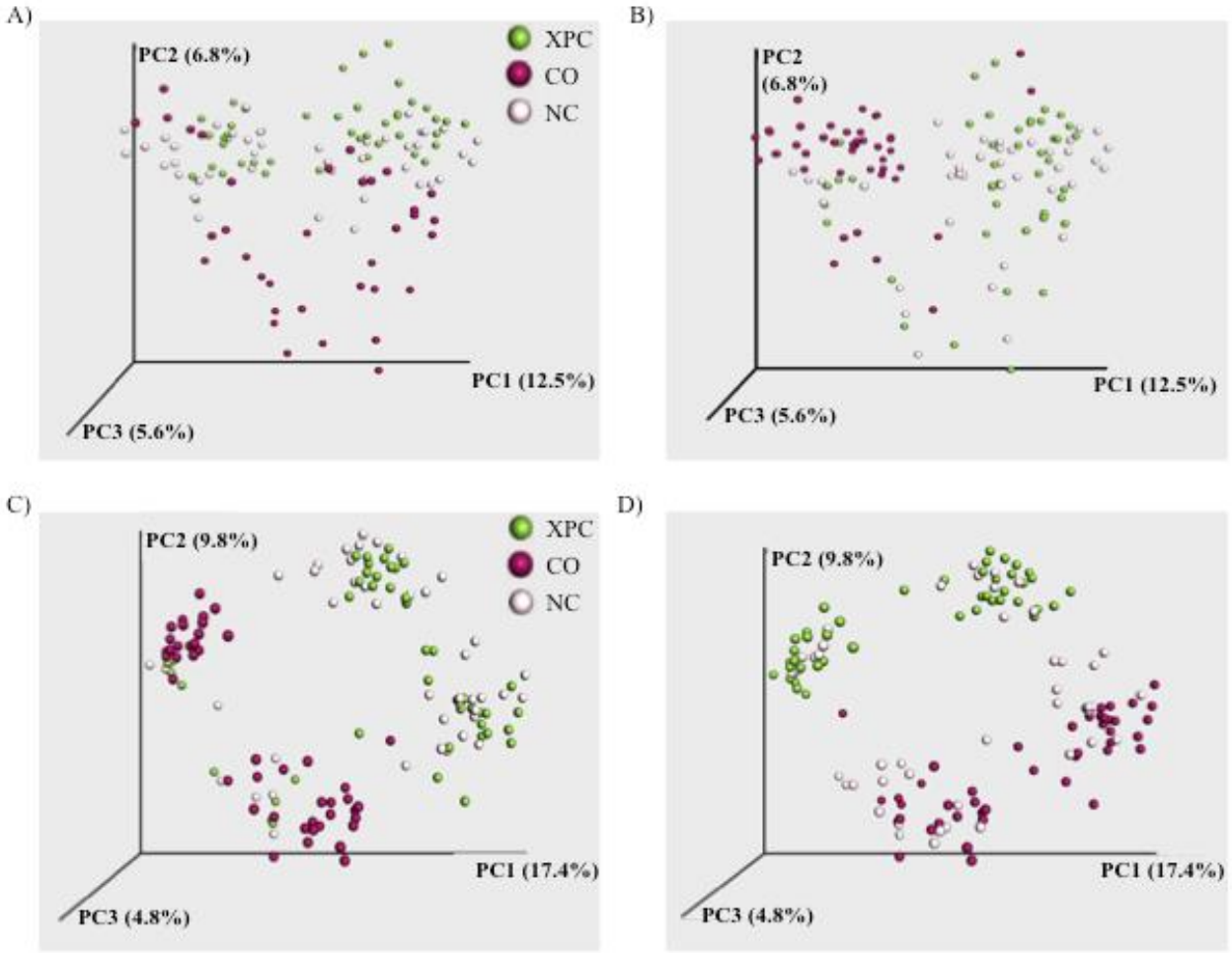


Figure 2 .9A to D: UniFrac unweighted PCoA plots of A) trial 1 based on treatment, B) trial 1 based on sampling age, C) trial 2 based on treatment, and 4) trial 2 based on sampling age.



3. Cecal microbiota analysis of feeding Original XPC™ to broilers raised under industry conditions

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Keywords: broiler, XPC, cecal microbiota, maturity, 16S rRNA

ABSTRACT

Biological supplements in poultry feed are of continued interest due to the improvements on growth performance, protection from pathogen invasion, and benefits on overall host health. The fermentation metabolites of Diamond V Original XPC™ (**XPC)** have previously been shown to improve commercial performance and reduce *Salmonella* in poultry. The current study sought to characterize the cecal microbiota using culture-independent analysis of the 16S rRNA gene in Coccivac-D sprayed broilers supplemented with XPC and/or Salinomycin (**SAL**). Ross 708 male broilers (n = 1280) were assigned to one of four possible treatments: Cocci-vaccine (**T1**), Cocci-vaccine + XPC (**T2**), Cocci-vaccine + SAL (in the grower diet only) (**T3**), and Cocci-vaccine + SAL (in the grower diet only) + XPC (**T4**). Analysis with a PCR-based denaturing gradient gel electrophoresis (**DGGE**) indicated a shift in the microbial populations present at the various sampling ages, 16, 28, and 42 d. Phylogenetic analysis indicated further consistency in microbial communities directly related to bird age. Identification of microbial communities present and the assessment of their respective quantities using Illumina MiSeq sequencing indicated treatment with XPC had no significant impact on microbial diversity (Shannon diversity index, Chao1 index, observed OTUs). Sampling age revealed significantly greater diversity at 28 and 42 d ($P < 0.05$) as compared to the 16 d for Shannon diversity index, while showing significantly increased richness and diversity in the 42 d sampling age (Chao1 and observed OTUs; $P < 0.05$). The results of the current study indicate that the chicken intestinal microbiota is impacted more by temporal changes rather than by the feed additive studied.

INTRODUCTION

Poultry researchers are attempting to find alternatives to antibiotic growth promoters (**AGP**) in poultry diets, which are being restricted for use in food animal production (HHS, 2015). The elimination of AGPs are due to concerns surrounding antibiotic resistance genes being transferred to humans through both the consumption of and contact with farm animals, rendering treatment with certain antibiotics ineffective (Witte, 1998). Various compounds have been and continue to be investigated for their ability to replace AGPs; one possible alternative is the fermentation metabolites of Diamond V Original XPC™ (**XPC**) (El-Husseiny et al., 2008; Kassem et al., 2012; Salim et al., 2013). The product XPC is a fermentation product that when fed in poultry diets has been shown to inhibit the survival of *Salmonella* while improving growth performance and health of poultry (El-Husseiny et al., 2008; Lensing et al., 2012; McIntyre et al., 2013; Carlson et al., 2016). A proposed mode of action for products similar in content to XPC (fermentation products) indicated the stimulation of diverse microbial communities in the gastrointestinal tract (**GIT**) of piglets (Kiarie et al., 2011).

The poultry GIT microbiome has elicited interest due to the potential health impacts it may have on the host (Brisbin et al., 2008; Hoffmann et al., 2009; Roto et al., 2015). The host-microbiome interactions may affect the host immune system, provide protection against epithelial damage, and increase nutrient availability and digestibility (Spring et al., 2000; Hooper et al., 2001; Brisbin et al., 2008; Jensen et al., 2008; Hoffmann et al., 2009). However, the poultry microbiome has a complex population of diverse microorganisms, the majority of which are of not-yet-identified species (Gong et al., 2002; Zhu et al., 2002; Ballou et al., 2016). Identification and/or development of feed additives that are capable of targeting the GIT microbiome and the specific microbial populations that have proven to be capable of improving

both health and growth performance would of great importance. Identifying the microbiome species and their respective abundances in a recognized mature and healthy poultry host would be the next logical step.

The objective of the current study was to identify the impact of feeding XPC on cecal microbial populations, and their relative abundances, present in the poultry GIT microbiome in response to treatment with XPC and/or Salinomycin (**SAL**). To eliminate known biases of culture-based methods, this objective was conducted using an Illumina MiSeq platform following preliminary data obtained via PCR-based denaturing gradient gel electrophoresis (**DGGE**).

MATERIALS AND METHODS

Experimental Design

A total of 1,280 day-old Ross 708 male broilers were sprayed with Coccivac-D (Merck, Kenilworth, NJ) and subsequently vaccinated (Marek's Infectious Bursal Disease, Newcastle Disease, and Infectious Bronchitis) at the hatchery. The birds were transferred to a grow-out facility and randomly assigned to one of four possible treatments as described in Table 3.1. There were 64 total floor pens used in this experiment, eight pens per block, eight blocks in total. The birds were assigned to pens (1.22 m x 1.22 m) using a randomization table; the density was set to that of commercial stocking density, 0.23 m^2. Each pen contained a Chore-Time feeder pan (Chore-Time Poultry Production Systems, Milford, IN) with a feed hopper above a reservoir and a nipple drinker (three nipples). Pens contained re-used pine shavings for bedding.

A pelleted three phase feeding plan was implemented including a starter (0 to 16 d), grower (16 to 28 d), and finisher (28 to 42 d) diet. Feed and water was provided *ad libitum*. The inclusion rate of XPC in the starter and grower diets (for the birds receiving XPC treatment) was 1.25 g/kg and subsequently reduced to 0.625 g/kg in the finisher diet. The inclusion rate for SAL

was a constant 0.044 g/kg in the grower diet only. The basal poultry diet contained no additional anti-coccidial or antimicrobial products prior to the treatment with SAL.

A review by the University of Arkansas Institutional Animal Care and Use Committee (**IACUC**) was exempt because the birds were raised in an off-campus commercial farm operation. The current study was restricted to microbiological evaluation of birds selected on site. The commercial cooperators used internal animal welfare protocols based on the National Chicken Council (**NCC**) guidelines (www.nationalchickencouncil.org).

Cecal DNA Extraction

At 16, 28, and 42 d, 24 birds (three birds per pen per treatment) were randomly chosen for cecal content removal. All cecal DNA extraction utilized the QIAamp Fast DNA Stool Mini Kit according to the manufacturer's manual (Qiagen, Valencia, CA) with the exception of using UltraPure DNase/RNase-Free Distilled Water (50 µl; Life Technologies, Carlsbad, CA) rather than using the provided buffer to elute DNA. Concentrations and purity measurements of the DNA were obtained with a Nanodrop ND-1000 (Thermo Scientific, Marietta, OH).

Preparation of DNA Samples for DGGE

Samples for DGGE analysis were pooled based on age and treatment group (each week of samples was separated into 16 pooled groups, 6 samples per group, 4 groups per treatment) to fit within the gel capacity. A polymerase chain reaction (PCR) was used to amplify the variable V3 region of the 16S RNA gene conserved region among bacteria; the primers used were described in Muyzer et al. (1993) (F: CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG; R: ATT ACC GCG GCT GCT GG). PCR amplification were performed in Peltier Thermal Cycler-200 (MJ Research, Inc., Waltham, MA) in reaction tubes (0.2 ml) containing 25 µl total volume: 15 µl JumpStart Taq ReadyMix (Sigma-

Aldrich, St. Louis, MO), 2000 nM forward primer, 2000 nM reverse primer, 1 µl of 50 ng DNA, 12 µl UltraPure DNase/RNase-Free Distilled Water (Life Technologies, Carlsbad, CA). The PCR analysis was performed with an initial denaturation step of 94°C for 5 min for DNA denaturation and held for 1 min. The temperature was subsequently decreased to 67°C for 45 s (-0.5/cycle), and extension at 72°C for 2 min; these steps were repeated for 17 cycles excluding the initial denaturation step. The program continued for 1 min at 94°C and 58°C for 45 s, and this sequence was repeated for 12 cycles. A final extension at 72°C for 7 min was performed; samples were held at 4°C. Negative controls using water were carried out for all amplifications performed. PCR amplification products were ensured via 1% agarose gel electrophoresis with ethidium bromide in 1X TAE buffer, stained, and visualized using a UV transilluminator; each gel run contained a 100 kb marker. Samples were diluted to 25 ng/µl in preparation for DGGE.

DGGE and Phylogenetic Tree Generation

The DGGE gels were cast with 8% acrylamide stock solutions and contained a gradient from 35 to 55% urea denaturant. Electrophoresis was carried out for 16 h at 55V and a temperature of 59°C using the DCode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA). After electrophoresis, the gel was stained using 20 µl of SYBR green in 650 ml 1X TAE on a shaker at 4°C for 40 min, and subsequently destained in distilled water for 10 min on a shaker at 4°C. Images of each gel were taken using a UV transilluminator to minimize the exposure of DNA bands to UV light exposure. DGGE gel images were uploaded to Quantity One software (Bio-Rad Laboratories, Hercules, CA) for phylogenetic tree analysis based on the unweighted pair group method with arithmetic mean (UPGMA) algorithm.

Library Preparation for Sequencing

Individual bird samples were utilized for sequencing unlike in the DGGE analysis, which used pooled samples. PCR was used to amplify the V4 region of the 16S rRNA gene with dual-indexed primers as described in Kozich et al. (2013). The primer mixture (F and R; 500 nM), DNA (10 ng/µl), and Accuprime Pfx SuperMix (Life Technologies, Carlsbad, CA) were combined in a 96 well plate, each plate contained one negative control (H_2O). PCR amplification was performed in Eppendorf Mastercycler pro S (Eppendorf) using the following PCR conditions: 95° for the initial 5 min, followed by a cycle of 95° for 30 s, 55° for 30 s, 72° for 1 min for 30 cycles, and ended at 72° for 5 min. PCR confirmation was conducted on 1% agarose gel of thirty randomly selected samples and one negative control from each plate.

Normalization of PCR amplification products was performed using Invitrogen SequalPrep kit (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol to remove any salts or free primers. Confirmation was performed on 1% agarose gel of thirty randomly selected samples and one negative control from each plate. The samples from each well in each plate were pooled together, with the exception of the negative controls. Pooled samples from each plate were run on 1% agarose gel, DNA was extracted from each gel, and the concentration was measured using a Qubit Fluorometer (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol.

Quantification of the pooled samples (each plate's pooled sample and all the plates' pooled samples combined) was conducted using both the Eppendorf realplex Mastercycler ep gradient S (Eppendorf, Hamburg, Germany) as well as the Agilent Bioanalyzer. KAPA Library Quantification Kit (KAPA Biosystems, Wilmington, MA) was followed according to the manufacturer's protocol ($R^2 = 0.999$; efficiency 96%). Amplicon lengths from the quantification were diluted to 4 nM. The sequencing library was combined with 0.2 N fresh NaOH and HT1

buffer to a concentration of 6 pM; PhiX Control was also prepared with 0.2 N fresh NaOH and HT1 buffer to a concentration of 6 pM; both the sequencing library and the PhiX Control were combined and loaded into the MiSeq reagent cartridge, along with the index primer and Reads 1 and 2 sequencing primers.

Sequence Analysis

Both of the demultiplexed R1 and R2 sequencing reads of approximately 250 bp in length were generated and downloaded from the Illumina Basespace website. Sequences obtained with fewer than 1000 reads were omitted from analysis and sequences were re-analyzed excluding these samples. The Quantitative Insights Into Microbial Ecology (QIIME ver. 1.9.0; Caporaso et al., 2010) pipeline was used to analyze sequences and classify into operational taxonomic units (OTUs) based on 97% sequence identity (Greengenes database used as the reference database) into the phylum, order, genus, and species taxonomic levels. Alpha and beta diversity generated data regarding observed OTUs, Chao1 index, Shannon diversity index, and weighted and unweighted UniFrac distance among samples, respectively.

Statistical Analysis

Data from growth performance were separated for comparison by treatment. Statistical analysis was conducted using JMP® Genomics (SAS Institute, Cary, NC) with means separated using LS Means ($P < 0.05$). The pen was the experimental unit for performance. Means of each data set from microbial prevalence were compared using ANOVA (one-way analysis of variance) test with a level of significance at 0.05. Species diversity and richness were assessed with QIIME by operational taxonomic units (OTUs), Shannon diversity index, and Chao1 (Chao, 1984; Zhang et al., 2015). A probability of less than 0.05 was considered significant. UniFrac

Principle Coordinate Analysis (**PCoA**) plots were generated through QIIME to illustrate the weighted and unweighted distances between samples based on sampling age.

RESULTS AND DISCUSSION

Analysis of Cecal Microbiota Shift Using PCR-Based DGGE

The objective of the current study included the investigation into the GIT microbiome, which has been attempted previously utilizing various methods. The culture-based methods used appear to be incomplete and selective in their results when considering the broader diversity of results obtained when using modern molecular and sequencing techniques (Bjerrum et al., 2006). Bjerrum et al. (2006) discusses the biases observed in culture-based methods that result in known bacterial species being identified and compares these methods to molecular methods, which revealed that only approximately 10% of the cecal bacterial species can be identified culturally, leaving the majority of species present as unknown (Salanitro et al., 1978; Mead, 1989; Ricke and Pillai, 1999; Apajalahti et al., 2004). Based on these biases, preliminary experimentation using DGGE was applied to assess the overall responses of the cecal microbial communities to various treatments and sampling ages. The technique of DGGE has been considered a reliable and relatively inexpensive pre-screening method for the quantification and diversity assessment of microbial populations present in an environment (Hanning and Ricke, 2011).

All amplification products generated from PCR were 233 bp in length. The microbiota analysis via DGGE indicated a shift in the presence and relative abundances (determined via band brightness) of microbial populations in response to the treatment as well as bird maturity. Pooled samples from DGGE of the 16 d samples indicated randomness in banding patterns, with no clustering among the treatment groups (Figure 3.1A), while 28 and 42 d samples exhibited increased successional consistency, both in the banding patterns and the brightness of the bands

(Figure 3.1B and C). This is also reflected in the phylogenetic tree that was generated in the clustering of samples according to treatment; there appeared to be approximately 73.5% homology among samples treated with XPC (T2 and T4) at the 42 d sampling age, as shown in Figure 3.1C. This enhanced banding stability directly related to bird maturity and treatment with XPC indicates a potential effect of XPC to accelerate the rate at which microbial communities reach mature levels in the ceca. Past and current literature, as reviewed by Schneitz (2005), asserts the beneficial impact of having a mature microbiome with robust communities of various microorganisms. A mature microbiome can allow for enhanced growth performance, pathogen control, reduced mortality, and overall health of the host (Patterson, 2011). Feed additives, such as XPC, that appear to be capable of supporting development of the microbial cecal populations to reach mature abundances at an earlier age may permit more productive bird growth throughout the production period with less pathogen infection (Brisbin et al., 2008; Patterson, 2011).

QIIME Analysis

Given the initial indication of microbial shifts occurring due to the treatment and age of the bird, a more detailed characterization of the microbial populations present and their abundances via the Illumina MiSeq platform was performed. Conducting both DGGE and sequence analysis allowed for comparative insight between the two methods. After filtering sequences using read quality and sample size requirements, there were a total of 280 samples (94 samples from individual birds at 16 d, 92 samples from 28 d, 94 samples from 42 d) of the V4 region of the 16S rRNA gene analyzed. Based on the Illumina Basespace, the analytical information regarding the sequences generated the following: 27,167,116 reads in total at a 1.49% error rate. The numbers of taxonomy groups identified (Edgar, 2010) were 19, 65, and 244 at the phylum, order, and genus levels respectively, when analyzed using Greengenes Core reference alignment (DeSantis et al., 2006).

Greater than 99% of the sequences from all the samples at all age points belonged to one of the five most populated bacterial phyla, namely Firmicutes (78.2%), Bacteroidetes (14.8%), Proteobacteria (3.5%), Tenericutes (1.9%), and Cyanobacteria (0.9%). The data analyzed at the phylum level indicated Firmicutes to be significantly greater than all other phyla among all treatments and all sampling ages except for XPC with SAL at 42 d (Figure 3.2A to D). Wei et al. (2013) found similar results to the current study in that chicken cecal microbiome samples comprised primarily of Firmicutes, Bacteroidetes, and Proteobacteria at approximately 70, 12, and 9% respectively. The abundances of Firmicutes among all samples at all sampling age points in the current study were approximately 70 to 80% (Figure 3.2A to D). Generally, Firmicutes are recognized for their production of butyric acid, which is associated with pathogen inhibition (den Besten et al., 2013). Bacteroidetes was revealed to be the second most abundant phyla among all treatments at each sampling age (Figure 3.2A to D). In contrast to Firmicutes observed abundances, the frequencies of Bacteroidetes were significantly decreased from the 28 to the 42 d sampling ages (Figures 3.2A to D). The less abundant phyla of the five (Proteobacteria, Tenericutes, and Cyanobacteria) revealed no significant differences across ages with the various treatments with the exception of Proteobacteria at the 16 d sampling age in T2 (Supplementary Figure 3.1A to C). Although there were no significant differences at the phylum level analysis among the treatments at each sampling age (Supp. Figure 3.1A to C), significant differences were observed when comparing the sampling ages of each treatment (Figures 3.2A to D), indicating a more temporal effect on the composition of the cecal microbiome. Oakley et al. (2014) also observed bird maturation to have greater influence on the cecal microbial populations than the addition of feed additives in the diet.

Similar to the phylum level, the five most abundant Order level microbial populations occurring among the treatments at each sampled time point with their total respective percentages were Clostridiales (75.8%), Bacteroidales (14.8%), Enterobacteriales (3.5%), RF39 (1.6%), and Lactobacillales (1.3%). In analyzing the order level microbial communities, as shown in Figures 3.3A to D, the microbial communities were not significantly different among treatments with the exceptions of Clostridiales at 16 d and Bacteroidales at 28 d, both being significantly decreased with treatment in comparison to the control. What is interesting in the Order level abundance analysis among age data is that Clostridiales significantly increased from 28 to 42 d sampling ages while Bacteroidales significantly decreased from 28 to 42 d sampling ages (Figure 3.3A to D).

Genus level evaluation of the cecal microbial populations indicated that eight abundant genera of bacteria were present for all sampling ages. *Faecalibacterium* and an unknown genus belonging to the *Enterobacteriaceae* family were present in samples at all three age points (Figure 3.4), decreasing in abundance with bird maturity. *Faecalibacterium*, a beneficial bacterium commonly observed in the both poultry and human intestinal tracts conferring anti-inflammatory properties and production of butyric acid, decreased as the birds matured (Miquel et al., 2013; Oakley et al., 2014). Presence and consistent abundance of *Oscillospira*, an unknown genus belonging to the *Ruminococcaceae* family, and an unknown genus of the Clostridiales family are evident throughout the sampling age points; all of these genera have been previously observed in a 16S rRNA- based analyses of the poultry cecal microbiota in an *in vitro* mixed culture assay (unpublished data). An unknown genus belonging to the *Rikenellaceae* family appeared to be in greatest abundance in 28 d samples when compared to both 16 and 42 d samples. Although these are recognized as beneficial bacteria, this observation of reduced

abundance with maturity may be aligned with the hypothesis that each bacterial community reaches a balanced and stable level in the microbiome (Patterson, 2011). Conversely, an unknown genus of the *Lachnospiraceae* family appeared to be lowest in abundance at 28 d, with higher abundances in both 16 and 42 d samples. *Bacteroides* abundance appears to be directly related to bird maturity. Bacteroidetes, the class to which *Bacteroides* belongs, is recognized to have a beneficial relationship with the host microbiome, allowing the ability to stimulate the host immune system to control competing microorganisms that may be present in the environment (Mazmanian et al., 2005; 2008).

Analysis at the species level identified 292 distinctive species being present in the cecal microbiota, however the majority (81.8%) of those species have not yet been identified in the reference database. The most abundant identified species, shared among all ages of birds, were *Faecalibacterium prausnitzii* (7.6%), *Bacteroides fragilis* (2.4%), *Blautia producta* (0.3%), *Gallibacterium genomosp.* (0.1%), and *Eggerthella lenta* (0.1%) (Figure 3.5), however there does not appear to be a correlation of species abundance with either dietary treatment or bird maturity. The bacterial species *F. prausnitzii* is recognized as a beneficial microorganism and is considered commensal among the microorganisms in the poultry GIT microbiome (Bjerrum et al., 2006). In healthy humans, *F. prausnitzii* comprises between 5 and 15% of the fecal microbiota and is a well-known producer of butyrate (Scupham, 2007; Flint et al., 2012; Miquel et al., 2013). Butyrate is identified as playing a major role in GIT physiology, allowing for protection from pathogen invasion as well as modulating the host immune system (Macfarlane and Macfarlane, 2011). *B. fragilis* has previously been isolated from chicken cecal contents and is a producer of both succinic and acetic acids (Salanitro et al., 1974). The remaining three identified species, although occurring at minimal percentages, were *B. producta*, *G. genomosp.*,

and *E. lenta*. Previously isolated from poultry, *G. genomosp*. appears to be pathogenic in nature and associated with mortality in various avian species (Christensen et al., 2003). The species *E. lenta* is also pathogenic, it is commonly observed in humans causing ulcerative colitis (Gardiner et al., 2014). Although *F. prausnitzii, B. fragilis, B. product, G. genomosp.,* and *E. lenta* were the most abundant identified species, there were many more (30 total) unidentified species comprising of much higher abundances among the most dominant species. This attests to the limited degree to which identification of bacterial species exists.

Evaluation of the observed OTUs and Chao1index indicated significant differences among the sampling ages of 16 and 28 d when compared to 42 d ($P = 0.003$; Figure 3.5A and B). The determination of numbers of OTUs and Chao1 index revealed no significant differences when comparing the various treatments (Figure 3.6A and B). The Shannon diversity index at the species level revealed significantly increased diversity at both 28 and 42 d sampling ages when compared to the 16 d sampling age (Figure 3.6A). However, comparison of Shannon diversity index among treatments revealed no significant differences (Figure 3.7B). PCoA plots based on sampling age generated from QIIME analysis indicated significant differences in both weighted and unweighted plots ($P = 0.001$; Figure 3.8A and B). There is sectioning with overlap among sampling ages in the unweighted PCoA plot, while tighter clustering with 42 d samples in the center. When comparing the PCoA plots from sequencing to the DGGE banding patterns, there are correlations linking OTU abundance to be the primary driving force behind clustering observed in both the weighted PCoA plot and the 42 d DGGE gels (Figures 3.8B and 3.1C, respectively).

Inclusion of XPC appears to allow for the microbial communities comprising the entire microbiome to reach their stable levels at an earlier age, as is indicated by DGGE analysis,

however this findings was less evident in the Next Generation Sequencing (NGS) data obtained. It has been suggested that the stable microbiome allows for increased digestion and nutrient absorption by the host GIT, in turn causing improved growth performance and feed conversion ratios (Brisbin et al., 2008; Hoffman et al., 2009; Delzenne et al., 2011). However, bird age appeared to be more influential on the species diversity, richness, and relative abundances, as is made evident by the Shannon diversity index, Chao1 index, observed OTUs, and weighted PCoA plots, respectively (Figures 3.5A, 3.6A and B, and 3.7B). There are several potential factors that may have affected the results observed, one being due to age in which the treatments were added as well as the decreased amount of XPC added in the diet from 28 to 42 d (0.625 g/kg) in comparison with 0 to 28 d (1.25 g/kg). A dosage effect of XPC has previously been observed to be influential on the growth performance and feed conversion ratio in poultry (El-Husseiny et al., 2008). The current results indicate the necessity for further research on matching the appropriate dosage to the age of the bird over the growth cycle for GIT bacterial selection and establishment of a mature GIT microbiota (Hanning and Diaz-Sanchez, 2015). An essential part to the improved growth performance as observed in AGP treated animals, is the establishment of stable bacterial populations that will not utilize all the nutrients, rendering them unavailable to the host (Hanning and Diaz-Sanchez, 2015; Roto et al., 2015).

The differences in results observed in DGGE when compared to NGS are potentially due to 1) heterologous sequences migrating to similar distances presenting banding patterns that would falsely allude to these sequences being phylogenetically related (Gafan and Spratt, 2005), 2) limited gel capacity thereby mandating the pooling of samples for large sample sizes (McCartney, 2002), 3) pooled samples for DGGE therefore potentially increasing background noise present due to many more samples being analyzed, 4) various PCR conditions have been

observed to inaccurately reflect the presence and abundance of certain taxa (Chandler et al., 1997), and 5) the hypervariable regions amplified in the current study for DGGE and NGS were V3 and V4 respectively. The hypervariable regions of the 16S rRNA gene chosen for amplification can greatly influence both the DGGE and NGS diversity profiles produced (Yu and Morrison, 2004; Chakravorty et al., 2007). The V3 region is typically used to identify taxa at the genus level, each band theorectically representing a unique genera of bacteria (Ercolini et al., 2001; Hanning and Ricke, 2011). Furthermore, there is a degree of underestimation of taxonomic richness and classification of lower accuracy when only partial sequences are used as compared to full or near full-length sequences (Yarza et al., 2014). Therefore, it would be beneficial to compare the data collected in the current study with full-length 16S rRNA gene sequences from a mature and healthy poultry cecal microbiota.

CONCLUSION

The exact mechanism of XPC is not yet defined and the current research does not support or refute the hypothesis of modulation of the GIT microbiome. In comparing the results from both methods of DGGE and NGS, the DGGE methodology appeared to indicate a potential modulation of the cecal microbial populations caused by the various treatments utilized in this research. This same observation became less apparent in the NGS analysis of the cecal microbiota in that the analysis indicated minimal detectable alteration among the treatments. However, DGGE and NGS both revealed age of bird to have the greatest influence in the alteration of the cecal microbiota. Similarly, the temporal changes in the analysis of the cecal bacterial populations have been indicated as influential factors on the modulation of the cecal microbiome in previous studies at 7, 21, and 42 days post hatch (Oakley et al., 2014). Lastly, the cecal microbiota analysis only allows for the identification of who is present but does not reveal

the corresponding metabolic activity associated with these microorganisms. Further research is necessary to evaluate the mechanism of how XPC effects growth performance, pathogen invasion, and overall health.

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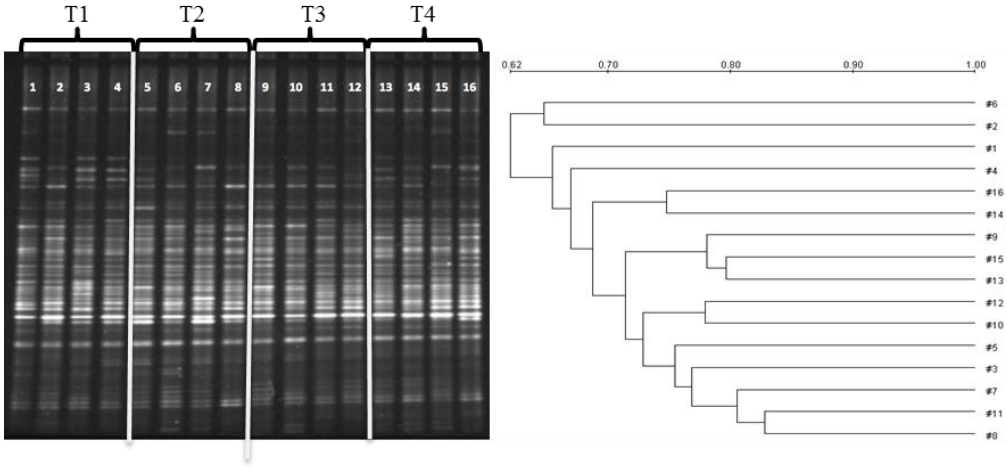
Table 3 .1: Experimental design describing the levels of Original XPC™ per diet (starter, grower, finisher) contained in each treatment.

		Original XPC™ (g/kg)		
ID	Description	Starter (0- 16 d)	Grower (16- 28 d)	Finisher (28- 42 d)
T1	Cocci-vaccine[1] (no XPC)	---	---	---
T2	Cocci-vaccine[1] + XPC[2]	1.25	1.25	0.625
T3	Cocci-vaccine[1] + SAL[3] (no XPC)	---	---	---
T4	Cocci-vaccine[1] + SAL[3] + XPC[2]	1.25	1.25	0.625

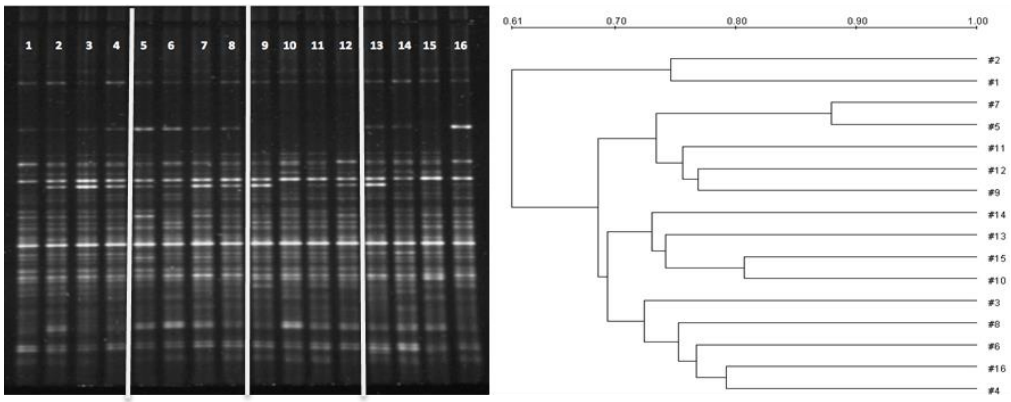
1. Coccivac-B (Merck, Kenilworth, NJ) sprayed at hatchery at 0 d.
2. Diamond V Original XPC™, Diamond V, Cedar Rapids, IA
3. Salinomycin sodium included only in grower diet (16-28 d) at 0.044 g/kg, Sacox, Huvepharma, St. Louis, MO

Figure 3 .1A to C: Pooled samples in DGGE and phylogenetic trees from A) 16 d, B) 28 d, and C) 42 d sampling ages including T1 to T4: DGGE (left) and phylogenetic tree (right). Lanes 1 to 4: T1 (Control) samples, lanes 5 to 8; T2 (XPC) samples, lanes 9 to 12: T3 (SAL) samples, and lanes 13 to 16: T4 (XPC + SAL) samples. Lanes from DGGE correspond to numbers labeled on the right side vertical axis of the phylogenetic tree.

A)



B)



C)

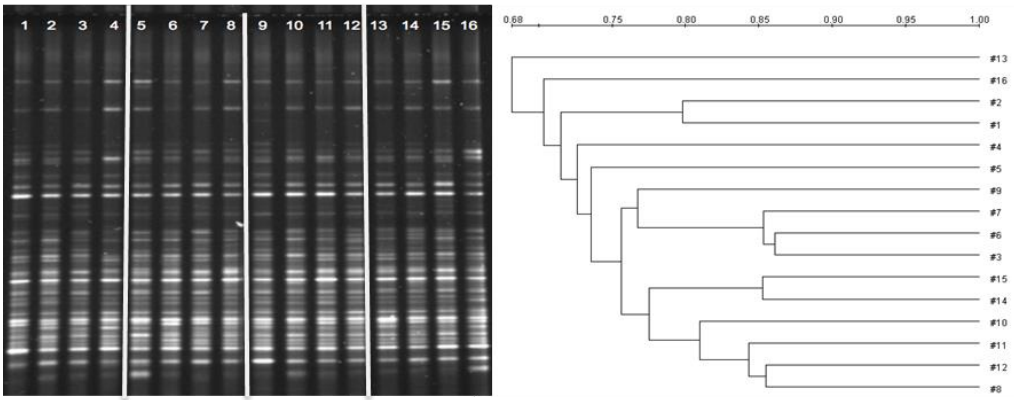
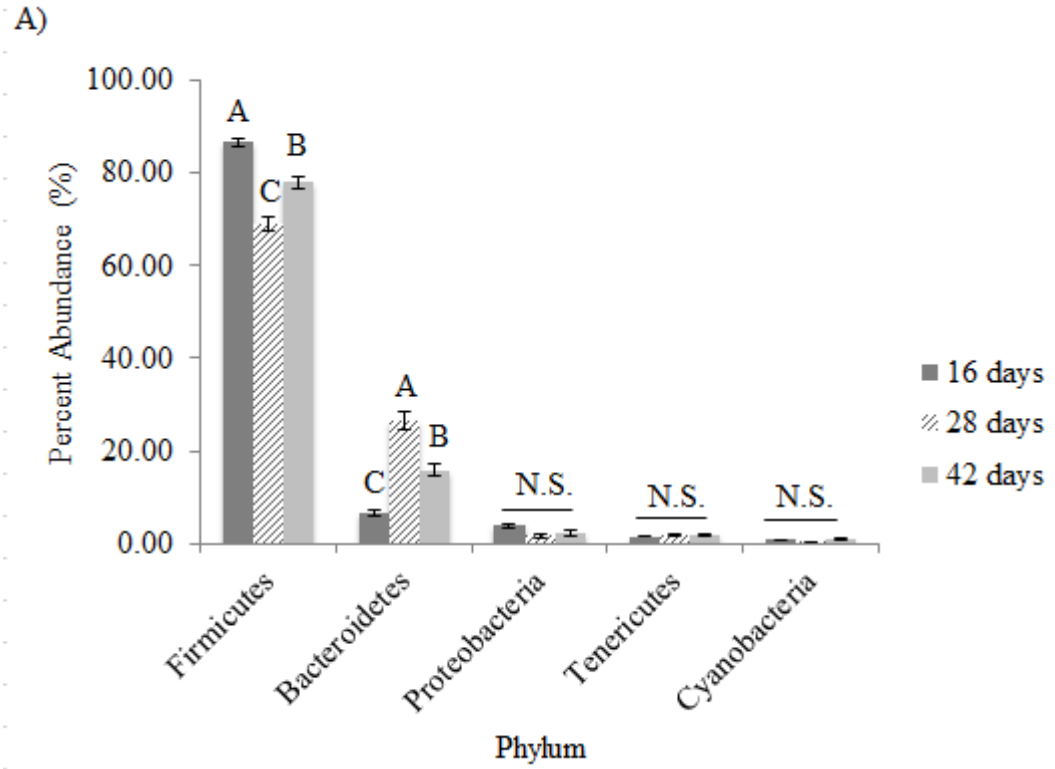


Figure 3 .2A to D: Phylum level abundance analysis among age sampling points for each treatment: A) Control, B) Salinomycin, C) XPC, D) XPC + Salinomycin. Treatment inclusion of Salinomycin occurred in the grower diet (16 to 28 d). Therefore there is only analysis of treatments containing Salinomycin at 28 and 42 d, while treatments containing XPC at all three sampling age points: 16, 28, and 42 d. Differing letters indicate significant difference ($P < 0.05$).



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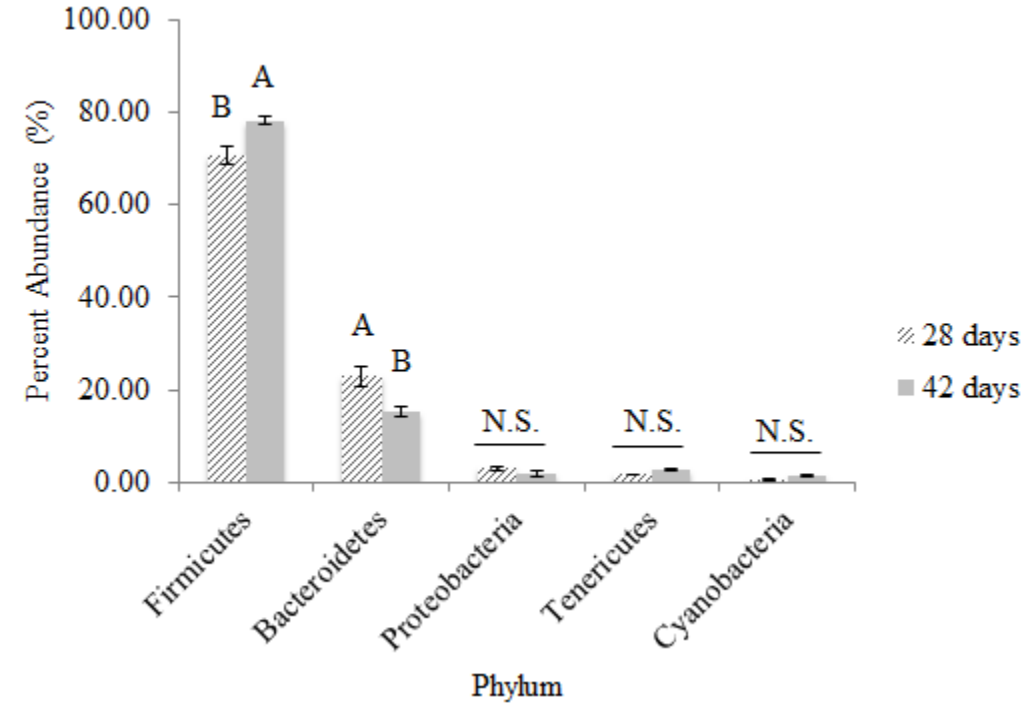
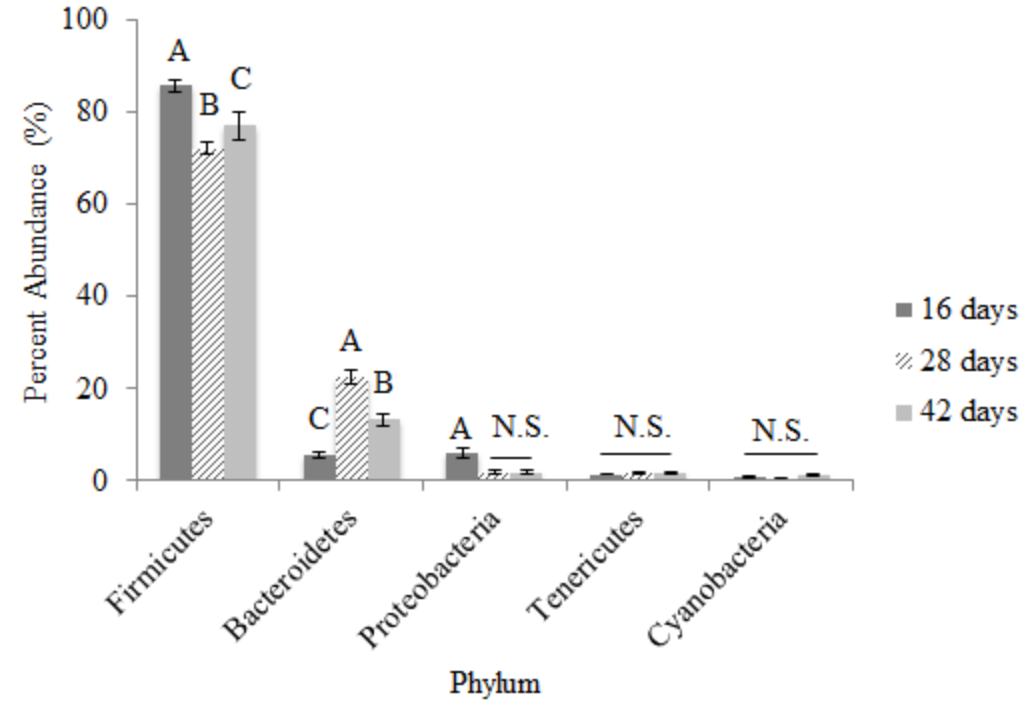

100.00
80.00
60.00
40.00
20.00
0.00
Percent Abundance (%)
B
A
A
B
N.S.
N.S.
N.S.
28 days
42 days
Firmicutes
Bacteroidetes
Proteobacteria
Tenericutes
Cyanobacteria
Phylum

C)


100
80
60
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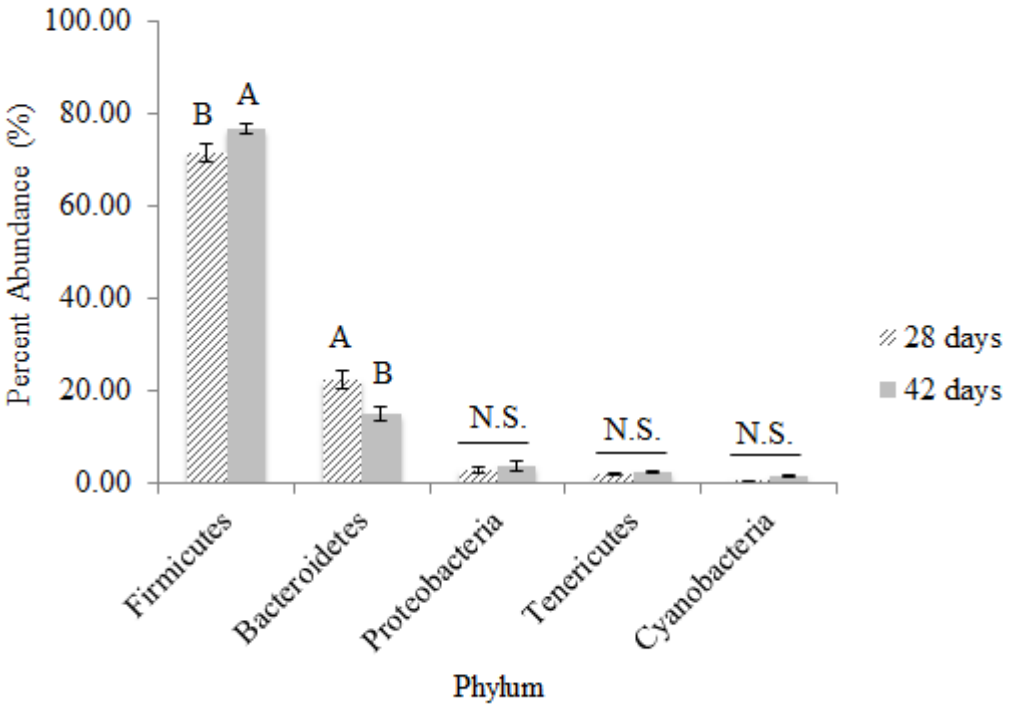
20
0
Percent Abundance (%)
A
B
C
C
A
B
A
N.S.
N.S.
N.S.
16 days
28 days
42 days
Firmicutes
Bacteroidetes
Proteobacteria
Tenericutes
Cyanobacteria
Phylum

D)


100.00
80.00
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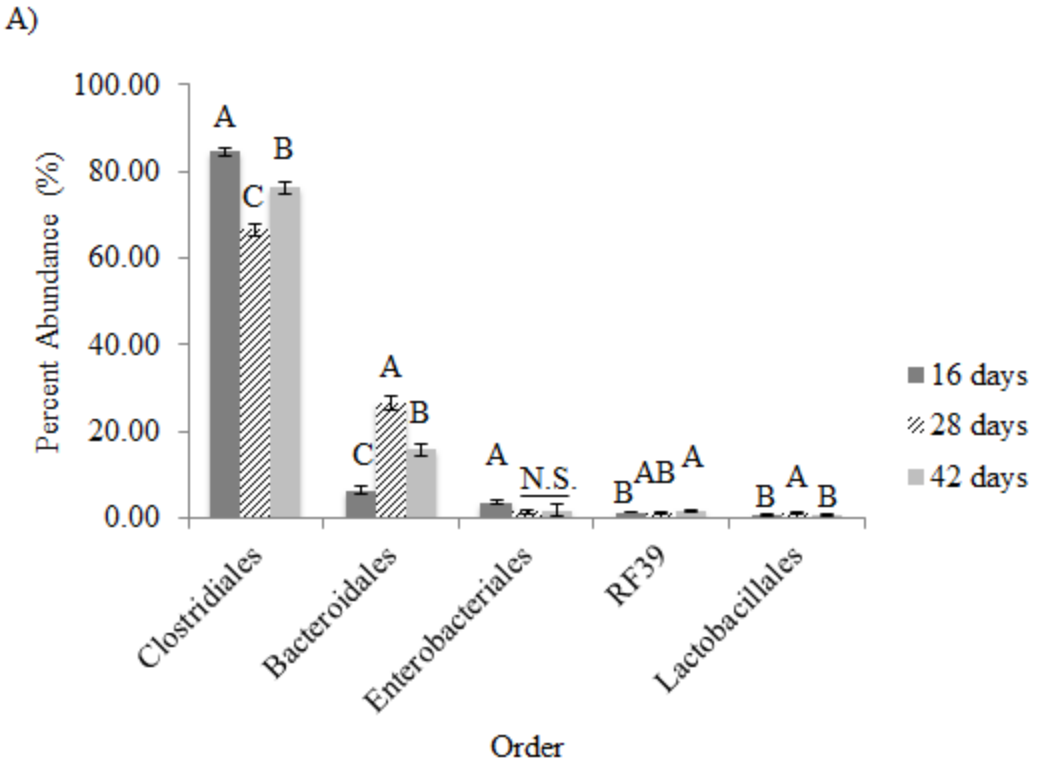
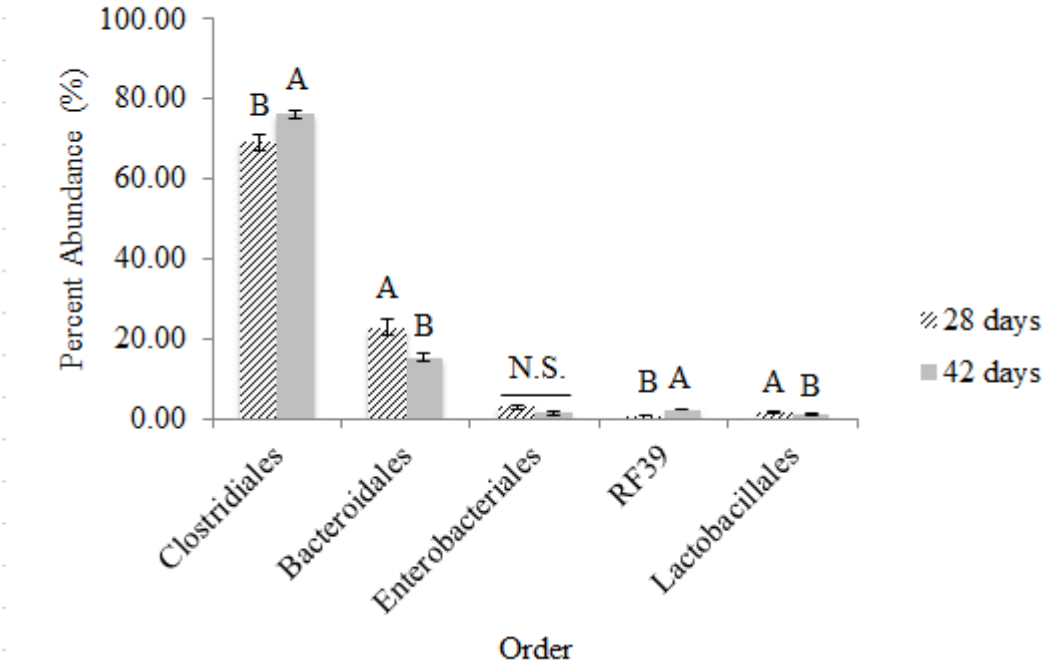
0.00
Percent Abundance (%)
B
A
A
B
N.S.
N.S.
N.S.
Firmicutes
Bacteroidetes
Proteobacteria
Tenericutes
Cyanobacteria
Phylum
28 days
42 days

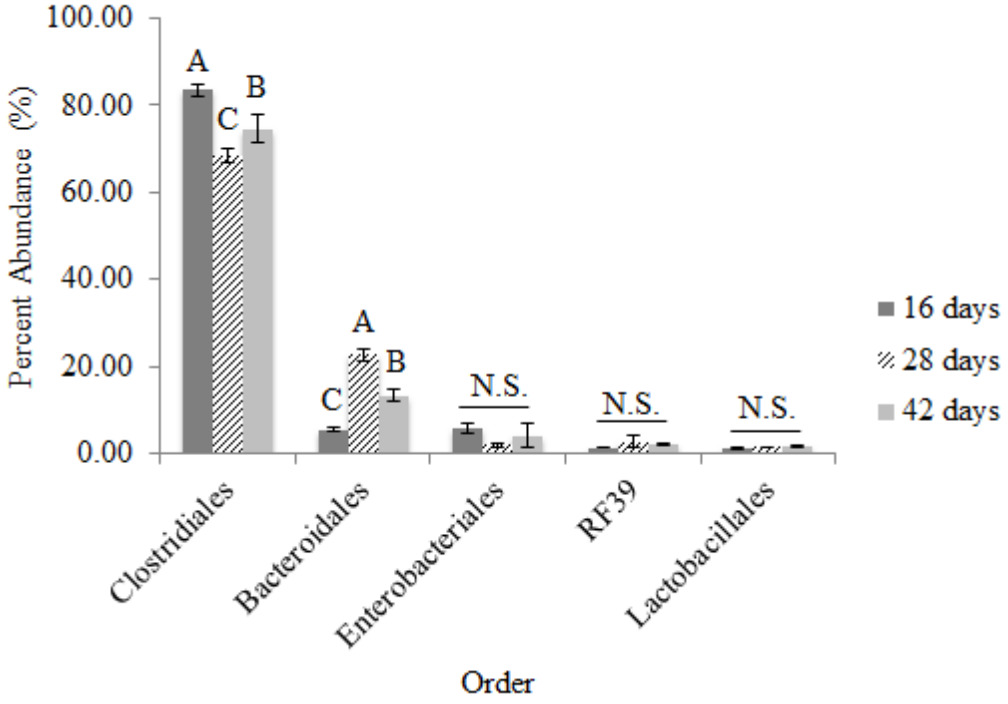
Figure 3 .3A to D: Order level abundance analysis among age sampling points for each treatment: A) Control, B) Salinomycin, C) XPC, D) XPC + Salinomycin. Treatment inclusion of Salinomycin occurred in the grower diet (16 to 28 d). Therefore there is only analysis of treatments containing Salinomycin at 28 and 42 d, while treatments containing XPC at all three sampling age points: 16, 28, and 42 d. Differing letters indicate significant difference ($P < 0.05$).



B)


100.00
80.00
60.00
40.00
20.00
0.00
Percent Abundance (%)
B
A
A
B
N.S.
B A
A B
28 days
42 days
Clostridiales
Bacteroidales
Enterobacteriales
RF39
Lactobacillales
Order

C)


100.00
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Percent Abundance (%)
A
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C
A
B
N.S.
N.S.
N.S.
16 days
28 days
42 days
Clostridiales
Bacteroidales
Enterobacteriales
RF39
Lactobacillales
Order

D)

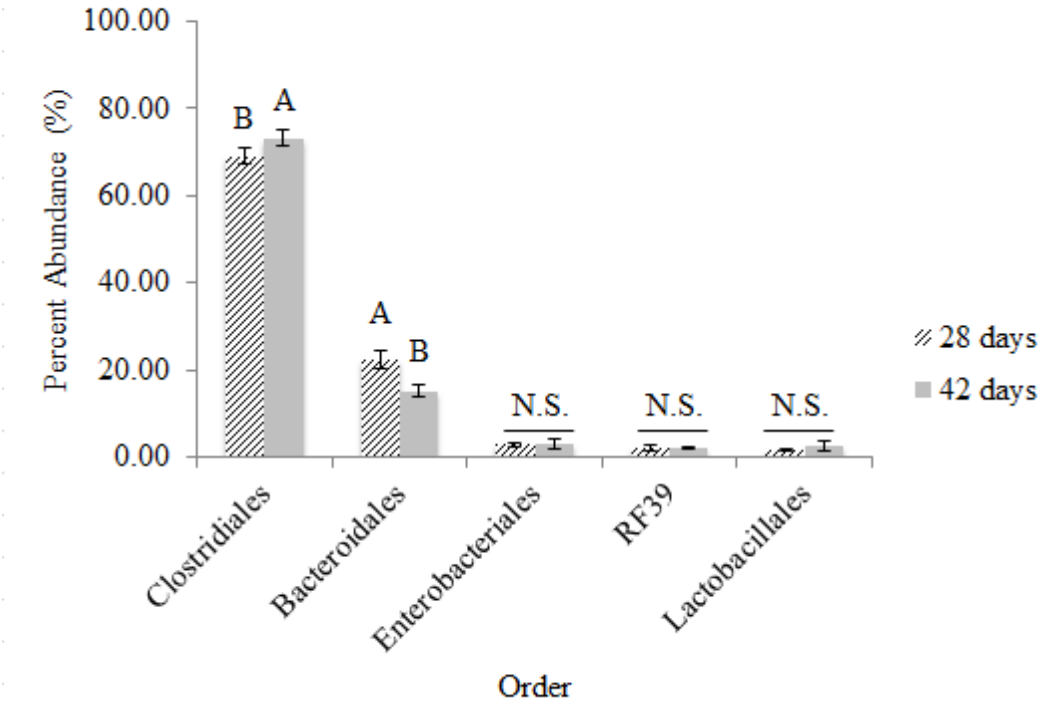

100.00
80.00
60.00
40.00
20.00
0.00
Percent Abundance (%)
B
A
A
B
N.S.
N.S.
N.S.
Clostridiales
Bacteroidales
Enterobacteriales
RF39
Lactobacillales
Order
28 days
42 days

Figure 3 .4: Genus level bar chart generated from QIIME analysis separated by sampling age. Colored font corresponds to genera abundance.

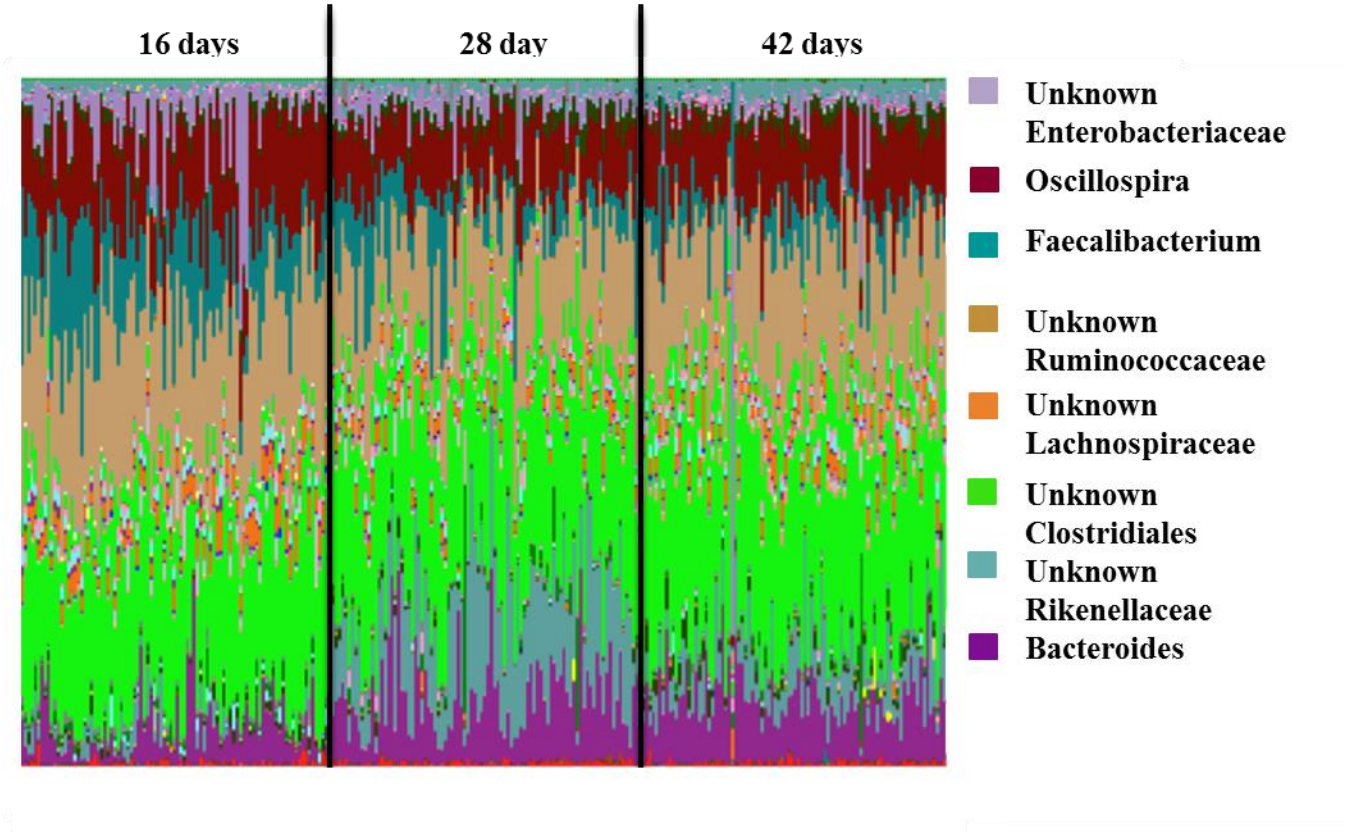


Figure 3 .5: Species level abundance analysis among age sampling points for each treatment Treatment inclusion of Salinomycin occurred in the grower diet (16 to 28 d). Therefore there is only analysis of treatments containing Salinomycin at 28 and 42 d, while treatments containing XPC at all three sampling ages: 16, 28, and 42 d (graphs identified as "Salinomycin" and XPC + Salinomycin" are identical to "Control" and "XPC" at 16 days, respectively).

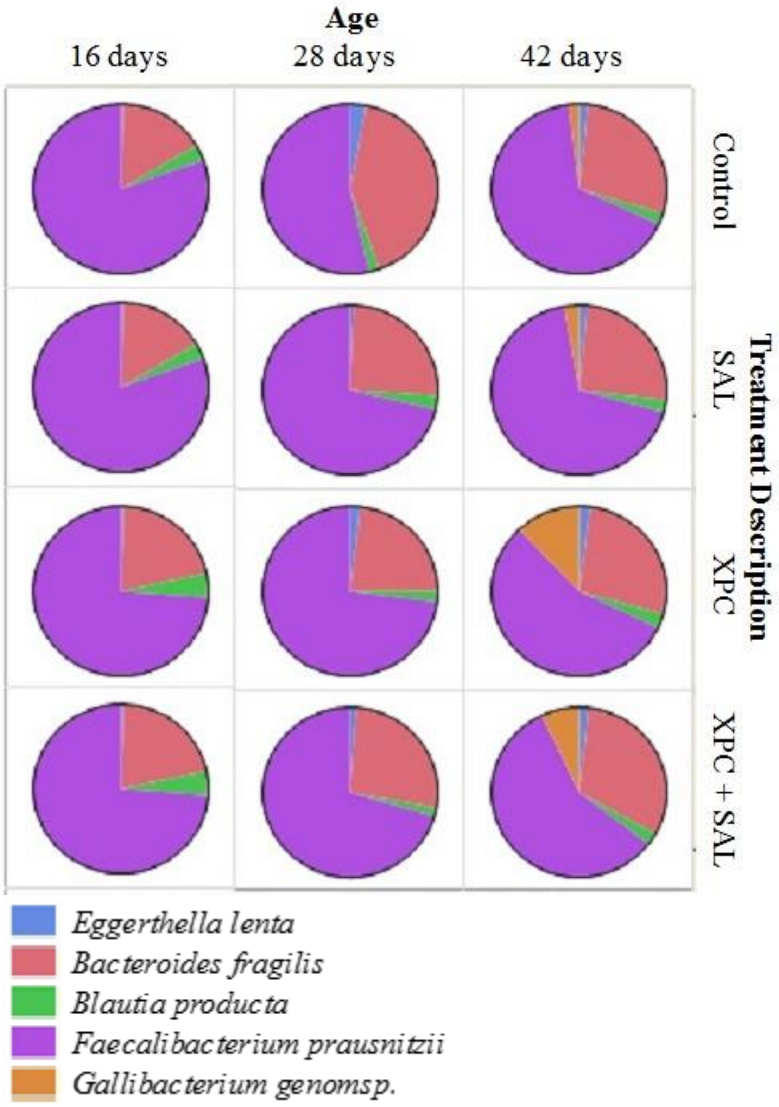


Figure 3 6A and B: Rarefaction curves for A) Chao1 indices and B) OTUs determined at the species level among all sampling age points (16, 28, 42 days). Statements of statistical significance are based on $P < 0.05$.

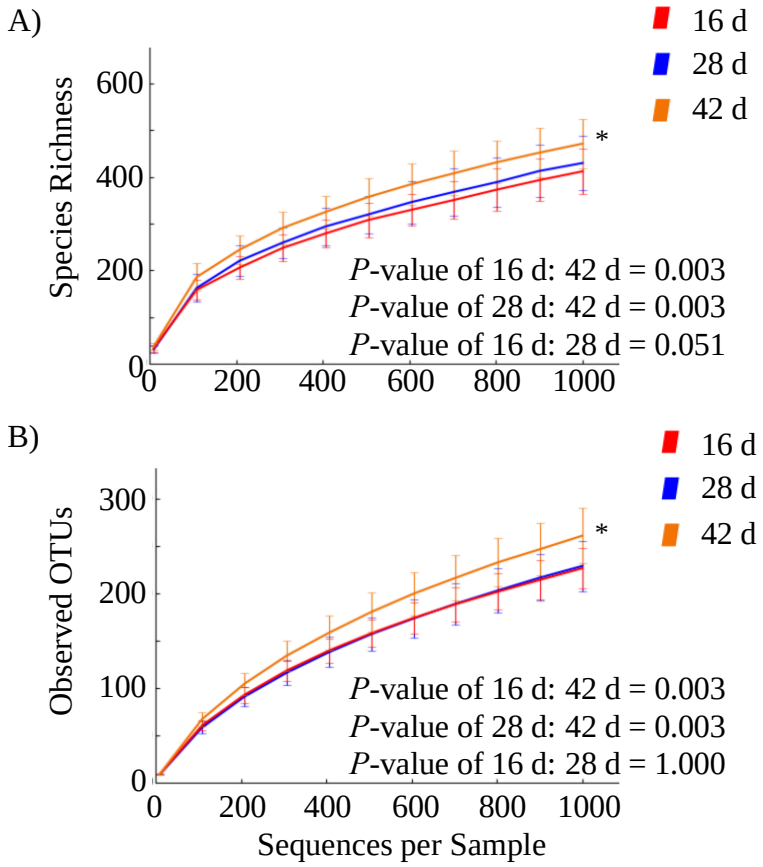


Figure 3 .7A and B: Mean Shannon diversity index determined at the species level comparing A) sampling age (16, 28, 42 days) and B) treatments (Control, Salinomycin, XPC, XPC + Salinomycin). Each marker indicates the mean Shannon diversity index; brackets represent the Mean ± SEM. Astericks (*) indicates a significant difference ($P < 0.05$).

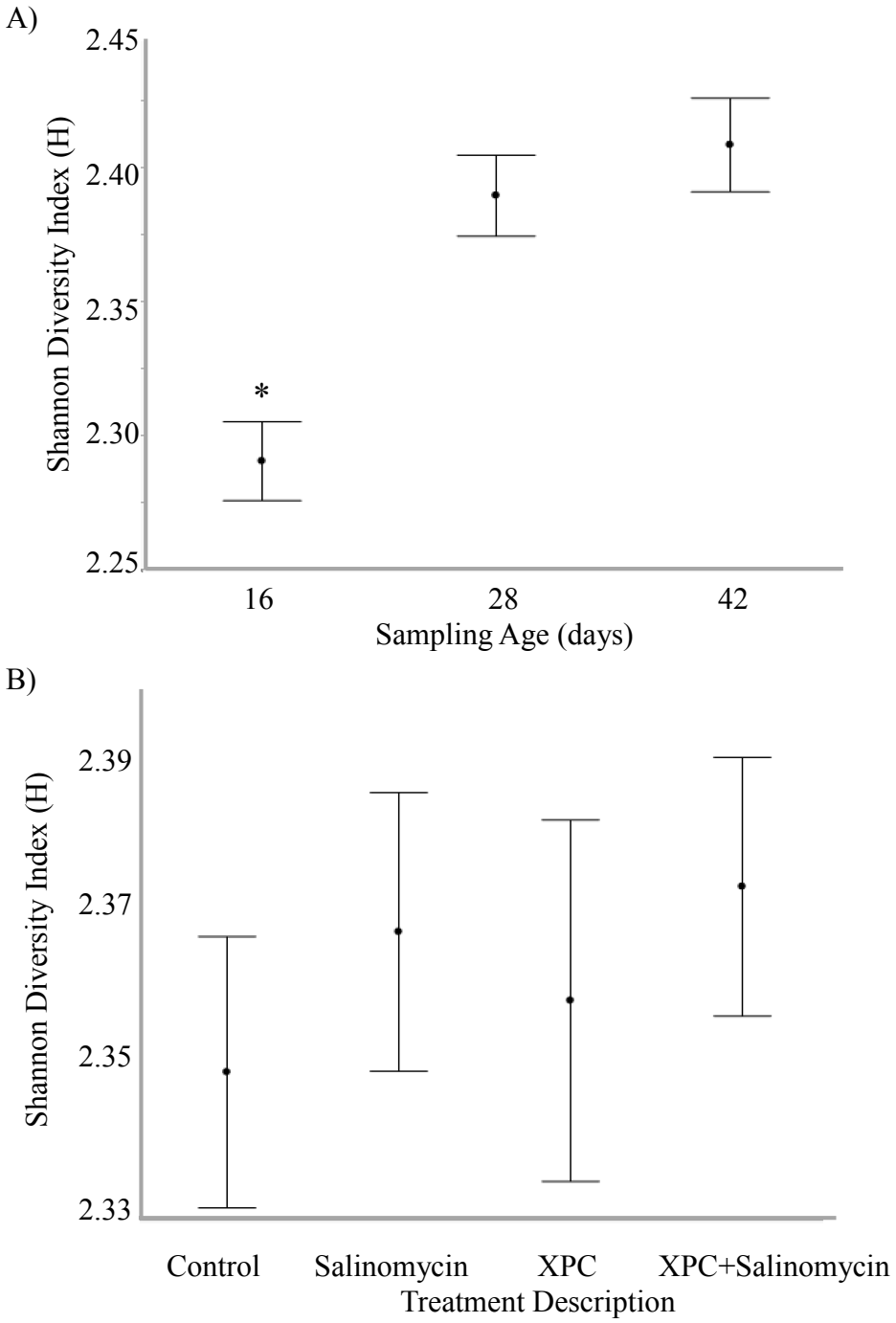
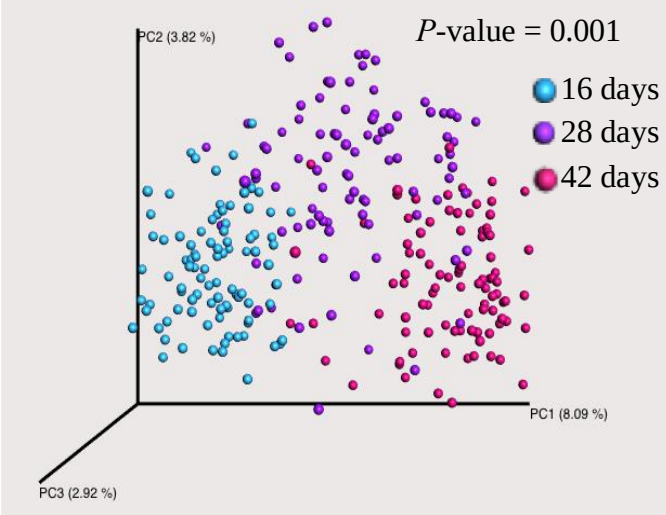
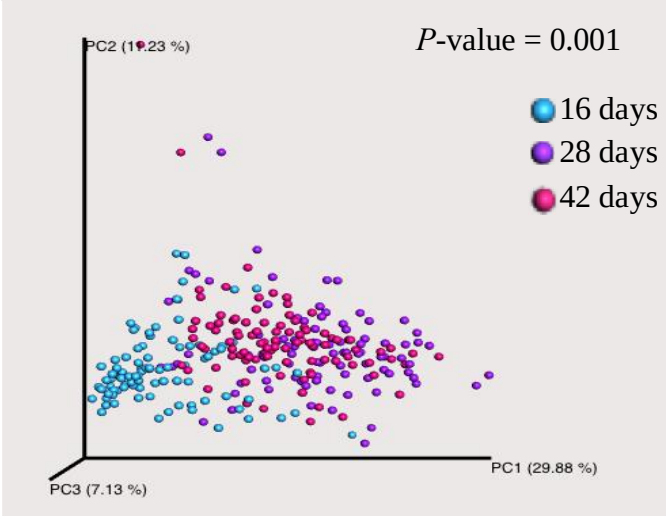


Figure 3 .8A and B: UniFrac PCoA plots A) unweighted and B) weighted plots of individual birds among sampling ages. Each colored dot is representative of a different sampling age. Statements of statistical significance are based on $P < 0.05$.

A)



B)



Conclusion

This thesis was focused on Original-XPC^{TM} (XPC), a product containing a mixture of fermentation metabolites. The objectives of this research were to observe the effects on *Salmonella* survival and the modulation of the intestinal microbiome using both an *in vitro* assay and an *in vivo* feeding trial. The results from chapter two (the *in vitro* assay) revealed XPC to allow for an accelerated rate at which *S.* Typhimurium survival is reduced within the cecal contents, regardless of variation among the microbial populations observed among the two trials presented (the variation observed among the trials is speculated to be due to environmental factors). In chapter three (the *in vivo* feeding trial), supplementation with XPC indicated phylogenetic differences via denaturing gradient gel electrophoresis (DGGE) associated with both the treatments and sampling ages, while the Illumina MiSeq sequencing results revealed significant differences only corresponding to sampling age. The analysis of the methods between the Illumina MiSeq and the DGGE indicated that although both characterize the microbial populations to different extents, the two methods have factors that limit the degree to which these methods are able to be directly compared (V3 vs. V4, pooled vs. individual samples, presence of background noise). In both chapters, treatment with XPC indicated little detectable influence on the intestinal microbiota, yet revealed the maturity of the cecal microbiome to increase the species diversity and richness, often associated with a healthy host. Lastly, this research illustrated the reliability of the *in vitro* assay utilized in the current research, as the *in vivo* feeding trial results were similar to the *in vitro* assay results.

Appendix

To Whom It May Concern;
I was given this email address by Dr. Mike Kogut to contact either Journal Manager, Jessica Kandbauer or the Journal Operations Assistant, Dimitri Christodoulou in regards to this matter:

I am writing to request permission to use the recently published literature review entitled *An introduction to the avian gut microbiota and the effects of the yeast-based prebiotic-type compounds as potential feed additives,* by Roto, S.M., Rubinelli, P.M., and Ricke, S.C. in my M.S. thesis. It was published in the Frontiers in Veterinary Sciences, Veterinary Infectious Diseases, under the research topic Gut Health: The New Paradigm in Animal Production.
I look forward to hearing back.

Thank you,
Stephanie Roto

Graduate Research Assistant
M.S. Candidate | Food Science | 2016

9/15/15

Frontiers in Veterinary Science Editorial Office <**veterinaryscience.editorial.office@frontiersin.org**>
to me

Dear Dr Roto,
Thank you for your message. Frontiers in a open access publisher and therefore no permission is needed should you wish to use your article for your thesis, provided the source is credited.

Under the Frontiers Terms and Conditions, authors retain the copyright to their work. All Frontiers articles are Open Access and distributed under the terms of the Creative Commons Attribution License, (CC-BY 3,0), which permits the use, distribution and reproduction of material from published articles, provided the original authors and source are credited, and subject to any copyright notices concerning any third-party content.

Please let me know if you have any further questions.
Kind regards,
Jessica Kandlbauer

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IACUC Exemption

FW: IACUC results

4 messages

Corliss Ann Obryan < cobryan@uark.edu> Thu, Nov 8, 2012 at 9:51 AM
To: Si Hong Park <parksh@uark.edu>

Si Hong,

Here is the email on the 2 projects not needing IACUC approval.

Corliss

From: Carol Ann Rodlun
Sent: Friday, November 02, 2012 3:14 PM
To: Steven C. Ricke; Kristen Elizabeth Gibson; Corliss Ann Obryan
Cc: Billy M. Hargis; Craig N. Coon
Subject: IACUC results

To All: After much discussion at the meeting and input from both Billy Hargis, who was familiar with the nature of your projects and Jason Apple, who was familiar with the Jeff Chewning's operation (he had high praise for it) ; it was decided to return the two protocols with a decision of " Of No Action Required of the IACUC" with the request, citing the following portion of the UAF Policy on Use of Animals in Research and Teaching "*There is one exception to this policy, which is that specific Animal Use Protocols shall not be required for agricultural teaching applications involving the non-stressful observation of farm animals, demonstration of judging techniques, demonstration of accepted farm management practices, or normal use of farm animals in production.* ***Instead, standard operating procedures detailing such practices and procedures shall be kept on file in the office of Research Support and Sponsored Programs [Office of Research Compliance] and of the Associate Vice President for Agriculture-Research, and shall be incorporated into the Policies and Procedures of the Dale Bumpers College of Agricultural, Food and Life Sciences and the Agricultural Experiment Station***", that you send a memo to the IACUC (which I will distribute to the Committee) that includes a Standard Operating Procedure for the transport and euthanasia of birds that will be taken as samples from the production birds set for these kinds of studies. The IACUC would like for you to consult with Dr. Hargis as to the preparation of this memo and the SOP and how best to proceed with these kind of projects in the future.

This has certainly been a learning experience for all of us!

IACUC Approval Letter: 13063

UNIVERSITY OF ARKANSAS

Office of Research Compliance

MEMORANDUM

TO: Steven Ricke

FROM: Craig N. Coon, Chairman

DATE: May 1, 2015

SUBJECT: IACUC Approval

Expiration Date: Jul 14, 2016

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your Modification to Protocol: 13063 Intestinal microbial profiles of young laying hens
(to increase animal number, extend termination date)

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond Jul 14, 2016 you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/aem

cc: Animal Welfare Veterinarian

Administration Building 210 • 1 University of Arkansas • Fayetteville, AR 72701-1201 • 479-575-4572
Fax: 479-575-3846 • http://vpred.uark.edu/199

IACUC Approval Letter: 15052

UNIVERSITY OF ARKANSAS

Office of Research Compliance

MEMORANDUM

TO: Steven Ricke

FROM: Craig N. Coon, Chairman

DATE: May 1, 2015

SUBJECT: IACUC Approval

Expiration Date: May 31, 2018

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your Protocol: 15052 Testing of Prebiotics for Control of Salmonella Colonization of the Chicken Intestine to begin June 1, 2015.

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond May 31, 2018 you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/aem

cc: Animal Welfare Veterinarian

Administration Building 210 • 1 University of Arkansas • Fayetteville, AR 72701-1201 • 479-575-4572
Fax: 479-575-3846 • http://vpred.uark.edu/199

IBC Approval Letter: 14003

UNIVERSITY OF ARKANSAS

Office of Research Compliance

July 11, 2013

MEMORANDUM

TO: Dr. Steven Ricke

FROM: W. Roy Penney
Institutional BioSafety Committee

RE: IBC Protocol Approval

IBC Protocol #: 14003

Protocol Title: "Intestinal microbial profiles of young laying hens"

Approved Project Period: Start Date: July 11, 2013
Expiration Date: July 10, 2016

The Institutional Biosafety Committee (IBC) has approved Protocol 14003, "Intestinal microbial profiles of young laying hens" You may begin your study.

If further modifications are made to the protocol during the study, please submit a written request to the IBC for review and approval before initiating any changes.

The IBC appreciates your assistance and cooperation in complying with University and Federal guidelines for research involving hazardous biological materials.

Administration Building 210 • 1 University of Arkansas • Fayetteville, AR 72701-1201 • 479-575-4572
Fax: 479-575-3846 • http://vpred.uark.edu/199